Cellular and molecular basis of the mouse oesophagus epithelial development

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CELLULAR AND MOLECULAR BASIS OF THE MOUSE OESOPHAGUS EPITHELIAL DEVELOPMENT

submitted by Wei-Yuan Yu

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Abstract

The mouse embryonic oesophagus is initially lined with simple columnar epithelium which changes during the course of development to stratified squamous tissue. In the present study, an *in vitro* model based on oesophageal explants isolated from E11.5d mouse embryos was developed. The columnar epithelial markers keratins 8 and 18 (K8, 18) were expressed at the beginning of the culture period and decreased in the basal epithelial layer at around day 5, but persisted in the suprabasal layers of the stratified epithelium for several more days. In contrast, the stratified squamous epithelial marker keratin 14 (K14) was absent at the beginning but progressively appeared in cells within the basal layer from 5 days of culture. The time course of the cell type transition seen in culture is similar to that *in vivo*. The two possible mechanisms for the change are either (1) a direct conversion of columnar cells to the basal layer cells of the squamous epithelium; or (2) an overgrowth of columnar by squamous cells. To investigate, first I demonstrated that some epithelial cells express both K8 and K14 by co-staining for both markers during the transition period *in vivo* and *in vitro.* Second, after electroporation of a construct containing the K14 promoter driving nuclear GFP into the epithelium of E15.5 oesophagus, some cells expressed both K8 and GFP. Third, there is no preferential loss of the columnar cells by apoptosis. Final, inhibitors of apoptosis and cell division do not affect the process. The results suggest that in normal development, the squamous epithelium arises from the columnar epithelium by a direct conversion process. In terms of the molecular mechanism, inhibitor studies suggest that *de novo* DNA methylation is required for the loss of the K8 expression but not for the acquisition of the K14 expression. Transcription factor *p63*, however, may play an important role in inducing the K14 expression.

List of Abbreviations

Acute phase proteins (APP)

Adenomatous Polyposis Coli (APC)

3-aminopropyltriethoxysilane (APTES)

Anterior intestinal portal (AIP)

Anterior-posterior (AP)

5-azacytidine (5-AzaC)

5-aza-2 deoxycytidine (5-Aza2C)

Barrett's metaplasia (BM)

Basal Medium Eagle (BME)

11-beta-hydroxysteroid dehydrogenase (11-β-HSD)

Bone marrow-derived cells (BMDC)

Bone morphogenic protein (BMP)

Bovine pituitary gland extract (BPE)

Casein kinase 1 (CK1)

CCAAT-enhancer binding proteins (C/EBPs)

Chromatin immunoprecipitation (CHIP)

Costal 2 (Cos2)

Cubitus interruptus (Ci)

Cytokeratins 1, 4, 5, 6, 8, 10, 13, 14, 16, 18 (K1, 4, 5, 6, 8, 10, 13, 14, 16, 18)

Decapentaplegic (Dpp)

delta-N (ΔN)

Developmental Studies Hybridoma Bank (DSHB)

3,3 -Diaminobenzidine (DAB)

4',6 -diamidino-2-phenylindole (DAPI)

1 ,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)

Disheveled (Dsh)

Dickkopf1 (Dkk1)

DNA methyltransferases (Dnmt)

E74-like factor 3 (Elf-3)

Electromobility shift assay (EMSA)

Embryonic stem (ES)

Enhanced green fluorescence protein (eGFP)

Epidermolysis Bullosa Simplex (EBS)

Epidermal growth factor (EGF)

European Collection of Cell Cultures (ECACC)

Fluorescence activating cell sorting (FACS)

Fluorescence isothiocyanine (FITC)

Fibroblast growth factor 2 (FGF2)

Foetal Bovine Serum (FBS)

Fumarylacetoacetate hydroxylase (Fah)

Fused (Fu)

Gastro-oesophageal reflux disease (GORD)

Glucagon-like peptide-1, 2 (GLP-1, GLP-2)

L-Glutamine (L-Glu)

Glycogen synthase kinase-3β (GSK-3β)

Granulocyte macrophage progenitors (GMP)

Gut-enriched Krüppel-like factor (GKLF)

Haematopoietic stem cells (HSC)

Haematoxylin and eosin (H & E)

Hairy and Enhancer of Split homologue 1 (Hes1)

Hank's Balanced Slat Solution (HBSS)

Hepatocyte growth factor (HGF)

Histone deacetylase (HDAC)

Indian hedgehog (Ihh)

Interleukin 3, 4 (IL-3, 4)

Involucrin (Inv)

Intestinal stem cell (ISC)

c-Jun N-terminal Kinase (JNK)

Keratinocyte serum-free medium (KSFM)

Lactase phlorizin hydrolase (LPH)

Lipoprotein receptor-related protein 5 and 6 (LRP5/6)

Mammalian achaete-scute homolog 1 (Mash1)

Mesenchymal stem cells (MSC)

Metalloproteinase matrilysin (MMP-7)

Minimum Essential Medium (MEM)

Mitomycin C (MitC)

Murine Maloney Leukaemia Virus (MMLV)

Musashi-1 (Msi1)

Myogenic regulatory transcription factors (MRFs)

Myosin heavy chain (MHC)

Myosin light chain (MLC2)

Neurogenin3 (Ngn3)

2-nitrophenyl-β-D-galactopyranoside (ONPG)

Periodic Acid Schiff (PAS)

Peroxisome proliferating activating receptor-gamma (PPARγ)

Phosphate buffer saline A (PBSA)

Post-SAM (PS)

Posterior intestinal portal (PIP)

Propidium iodide (PI)

Protein kinase A (PKA)

Relative luciferase unit (RLU)

Reverse osmosis (RO)

Reverse transcription – Polymerase Chain Reaction (RT-PCR)

Scanning electron microscope (S.E.M.)

Skeletal muscle myosin (FAST) (SkMyo)

Small proline-rich protein family (SPRR)

alpha-Smooth muscle actin (SMA)

Smooth muscle heavy chain myosin (smMHC)

Smooth muscle myosin kinase (SmMyoK)

Sonic hedgehog (Shh)

Sterile alpha motif domain (SAM)

Sucrase-isomaltase (SI)

Suppressor of Fused (SUFU)

Tetramethylrhodamine isothiocyanate (TRITC)

Texas Red (TR)

Transactivating (TA)

Transit amplifying (TA)

Tumour necrosis factor-α (TNF-α)

5 Untranslated region (5 UTR)

Uridine diphosphate glucuronosyltransferase (UDPGT)

Wingless (wg)

5-bromo-4-chloroindol-3-yl β-D-fucopyranoside (X-Fuc)

Contents

Chapter 1 Introduction

1.1 Metaplasia and transdifferentiation, definitions and importance in development and disease

1.1.1 What is transdifferentiation?

The conversion from one cellular phenotype to another is termed metaplasia, and can include conversions between stem cells of different tissue types (Tosh and Slack, 2002). Transdifferentiation is a subclass of metaplasia and by definition it is an irreversible switch of one already differentiated cell to another type of differentiated cell (Eguchi and Kodama, 1993). Some pathologists use the term "metaplasia" to describe cell-type replacement from an overgrowth of one population by another, when it is the result of cell migration and outgrowth. According to Eguchi and Kodama, two important experimental criteria need to be established for a process to be defined as transdifferentiation. Firstly, the two different phenotypes (i.e. the cells that are present before and following the transdifferentiation event) should be clearly defined. Both morphological appearances and molecular or biochemical evidence is usually required. Secondly, the cell lineage (ancestor-descendent) relationship between the two cell types needs to be established. Other questions of interest involving metaplasia and transdifferentiation are whether (1) cell division is involved and (2) the transition is between closely related developmental hierarchy pathways or from distant cell lineages (Slack and Tosh, 2001).

1.1.2 Master regulatory genes

Transdifferentiation is normally found to occur in cells that have the same embryonic origin (e.g. pancreas and liver are both derived from the endoderm)

(Slack, 1985; Slack and Tosh, 2001; Tosh and Slack, 2002). It is possible that there is just one or a few important regulatory gene(s) known as master switch genes that are important in deciding the fate of a cell. When master switch genes are mutated or misexpressed either ectopically or as a result of a disease process, transdifferentiation can occur (Fig 1.1).

A number of genes that exist exemplify the definition of the master switch gene. These include MyoD for myogenesis, peroxisome proliferating activating receptor-gamma (PPARγ) and CCAAT-enhancer binding proteins (C/EBPs) for adipogenesis, NeuroD for neurogenesis and PHA-4/FoxA for pharyngeal organogenesis in *C. elegans*.

The muscular master switch gene MyoD was found by a unique experimental approach. A mouse fibroblastic progenitor cell line-10T1/2 derived from C3H mouse embryonic cells (Reznikoff et al., 1973), was found to produce three different mesenchymal cell lineages when treated with the hypo-methylating agent-5-azacytidine (Taylor and Jones, 1979; Konieczny and Emerson, 1984). The three lineages were striated muscle cells, lipid-storing adipocytes and cartilage-producing chondrocytes. The phenotypes persisted after withdrawal of 5-azacytidine, suggesting a stable switch had occurred. To try and identify which genes were responsible for the switch in phenotype, a genomic DNA library was isolated from 5-azacytidine-treated 10T1/2 and screened by transfection into the untreated fibroblast (Davis et al., 1987), only 1 in 15,000 transfected colonies was found to result in myogenic conversion, indicating the a single or only a few loci in the genome is sufficient for this conversion. Later, myoblastic-specific subtracted cDNA probes generated from the proliferating myoblasts were used for screening and one gene – MyoD was found to be

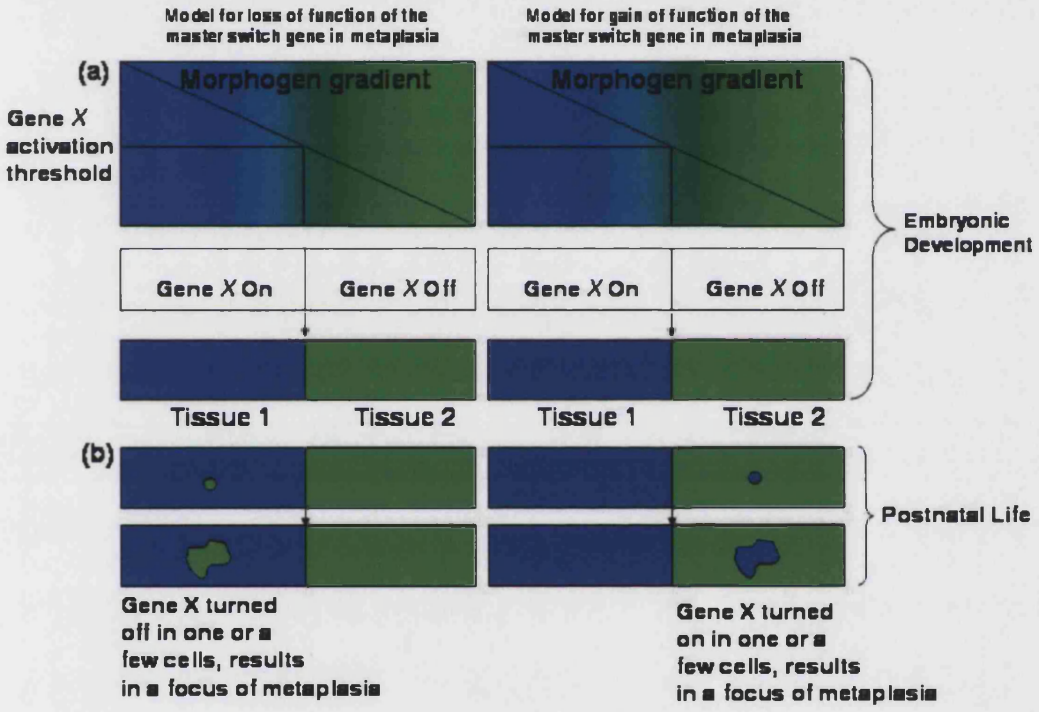

Model for loss of function of the master switch gene in metaplasia
Model for gain of function of the master switch gene in metaplasia
(a)
Gene X activation threshold
Morphogen gradient
Morphogen gradient
Gene X On
Gene X Off
Gene X On
Gene X Off
Embryonic Development
Tissue 1
Tissue 2
Tissue 1
Tissue 2
(b)
Postnatal Life
Gene X turned off in one or a few cells, results in a focus of metaplasia
Gene X turned on in one or a few cells, results in a focus of metaplasia

Fig 1.1 Model for a master switch gene in metaplasia.

A metaplasia model involving the master switch gene. (a) At some point during embryonic development, neighbouring tissue differs in expression of just one master switch gene *X*. The expression of gene *X* may be controlled by a certain threshold of a morphogen gradient. Gene X subsequently controls the identity (expression profile of tissue specific genes) of the two neighbouring tissues. (b) In postnatal life, somatic mutation, change in environmental stimuli or loss of epigenetic regulation in one or some (progenitor) cells may cause gene X to be turned off in a gene X expressing tissue (Loss of function model), or gene X to be turned on in a tissue originally not expressing gene X (Gain of function model). This will cause a focus of metaplastic to occur. (Figure adapted from Tosh and Slack, 2001; Shen et al., 2003).

responsible for the change of 10T1/2 cells to the muscle phenotype (Lassar et al., 1986; Davis et al., 1987). It is now known that there are at least four myogenic regulatory transcription factors (MRFs) that are important for skeletal muscle commitment and myotube formation - Myf5, MyoD, myogenin and MRF4, and they are all basic helix-loop-helix nuclear proteins (Parker et al., 2003). The DNA-binding domain of MyoD is similar to the proto-oncogene, c-myc (Miner and Wold, 1991) and MyoD has been postulated to compete for c-myc binding sites to suppress cell division and induce muscle progenitor cells to differentiate. This was the first found example of a master regulatory gene, which is able to transdifferentiate cells such as fibroblasts that are originally not muscular to adopt a muscle phenotype (myotube formation and fusion) and express specific muscle cellular markers, e.g. myosin heavy chain (MHC) and myosin light chain (MLC2) (Weintraub et al., 1989). What is more spectacular is when MyoD is introduced into some other cell lines, such as primary fibroblasts, adipocytes, smooth muscle cells, hepatocytes, baby hamster kidney cells, melanoma cells or neuroblastoma cells, the cells start to express a muscle phenotype. Remarkably, this conversion crosses the germ layer boundaries. Muscle, fibroblasts, adipocytes, smooth muscle and kidney cells are mesoderm in origin, but hepatocytes are endodermal, and melanoma and neuroblastoma are ectodermal. However, not all cells forced to express MyoD could turn on the muscle differentiation programme. Expression of MyoD in CV1 (an African green monkey kidney-derived cell), HeLa (human cervical carcinoma), and a human hepatoma cell line-HepG2 failed to activate differentiated muscle markers, such as desmin or MHC. In these experiments, the authors found co-expression of the parental neural or melanocyte markers with the muscle markers, but did not find the co-expression of adipocyte

markers with the muscle markers in the transdifferentiated myocytes. The explanation is possibly because myogenesis might be exclusively inhibiting other differentiation pathways that are very close to the muscle lineage, but not those that are further away from the developmental pathway. The understanding of transdifferentiation therefore provides insights to the normal embryonic development.

Myocytes can also be induced to transdifferentiate into adipocytes. Normally, G8 myoblasts do not express the adipocyte specific transcription factor: Peroxisome proliferator activated receptor-gamma (PPARγ) and CCAAT enhancer binding proteins (C/EBPs). When PPARγ and C/EBPα are ectopically expressed in G8 cells, the myogenesis programme is inhibited under normal myogenic-promoting culture conditions (Hu et al., 1995), and expression of MyoD, Myf-5, myogenin and MRF-4 are markedly reduced. On the other hand, markers specific for adipocytes, such as aP-2, adipsin, LPL and PEPCK appeared only in G8 myoblast line co-expressing both PPARγ and C/EBPα, and most of the cells become Oil red O-stained positive, which marks lipid storage within the cells. It was also found that C/EBPβ and C/EBPδ stimulates adipogenesis in fibroblasts (Yeh et al., 1995), which probably occurs through up-regulation of PPARγ expression (Wu et al., 1995). However, the normal activation of the PPARγ nuclear receptor in adipogenesis still requires ligand binding (Wu et al., 1996). There are also cases where over-expression of C/EBPα in 8 different lines of fibroblastic cells (such as NIH3T3, BALB/c-3T3) promotes adipogenesis (Freytag et al., 1994), but this only occurs when large quantities of C/EBPα is expressed. Normally, C/EBPα is expressed later than PPARγ in preadipocyte differentiation, and physiological amounts of C/EBPα can synergize with PPARγ in promoting adipogenesis in fibroblasts (Tontonoz

et al., 1994). It is also found that the canonical Wnt signalling pathway is involved in inhibiting the pre-adipocyte differentiation into adipocytes (Ross et al., 2000). The inhibitory mechanism does not affect the expression of C/EBPβ and C/EBPδ, but controls the expression of PPARγ, C/EBPα and the further activation of adipogenesis; therefore, Wnt signalling is thought to suppress the differentiation of pre-adipocytes by acting downstream of C/EBPs. Cross-regulation of C/EBPs and PPARγ is important in maintaining the adipocyte differentiated state and the presence of both transcription factors is powerful enough to change the muscle cell lineage into fat cell lineage (Rosen et al., 2000). A simplified diagram of transcription factors important in adipogenesis is shown in Fig 1.2.

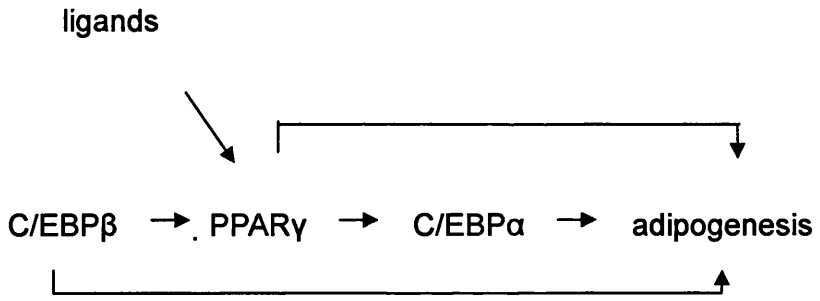


Fig 1.2 A simplified diagram below shows the transcriptional regulatory hierarchy in the process of adipogenesis (arrows indicate activation or up-regulation).

A well understood "master switch" transcription factor controls the identity of *C.elegans* pharynx. PHA-4 is a *C.elegans* homologue of the mammalian forkhead transcription factor *FoxA/HNF3* gene (Mango et al., 1994; Kalb et al.,

1998). Nearly all of the pharyngeal-specific genes necessary for the foregut to develop into the pharynx contain binding sites for *PHA-4/FoxA*. It is the copy number of the *PHA-4/FoxA* binding element in the enhancer region that controls the timing and location of pharyngeal gene expression (Gaudet and Mango, 2002; Ao et al., 2004). *PHA-4* also interacts with different genes such as *DAF-12/NHR* to further finetune which pharyngeal genes are to be appropriately expressed (Gaudet and Mango, 2002). However, there is no evidence at present to suggest that ectopic expression of *PHA-4/FoxA* will lead other cell types to transdifferentiation into pharyngeal cells.

1.1.3 Transdifferentiation of pancreas and liver

In our lab, we have worked extensively on the transdifferentiation of the pancreas and liver.

(1) The rat pancreatic cell line, AR42J-B13, upon treatment with the synthetic glucocorticoid - dexamethasone, will lose the expression of the pancreatic markers, e.g. amylase, and begin to express liver markers such as glucose-6-phosphatase, albumin, transferrin, glucokinase, CYP3A1, CYP2B1/2, Uridine diphosphate glucuronosyltransferase (UDPGT) and aryl-sulfotransferase (Shen et al., 2000; Tosh et al., 2002). When C/EBPβ, a transcription factor not usually found in pancreas, is ectopically expressed in AR42J-B13 cell line, the cells transdifferentiates and express C/EBPα and perform liver-specific functions such as the production of acute phase proteins (APP) (Kurash et al., 2004). This ability to induce transdifferentiation of pancreatic cells to hepatocytes indicates the master regulatory function of C/EBPβ in the transdifferentiation of AR42J-B13 into liver cells. Primary culture of hepatocytes usually results in loss of the

hepatocyte differentiated properties. One possible role for the B13 transdifferentiated hepatocyte model is as an alternative to rodent hepatocytes for studying of liver function *in vitro*. The B13 cells were also found stably expressing differentiated markers of the hepatocytes after long term dexamethasome treatment.

(2) In the reverse situation, a Xenopus liver can be converted into exocrine (amylase-expressing cells) and endocrine pancreatic tissue (such as insulin-secreting cells). When a modified Xenopus version of the pancreatic gene Pdx1 fused in frame with VP16 (the co-activator domain derived from Herpes simplex virus) was introduced into the embryonic liver by transgenesis, ectopic pancreas could be found in hepatic regions of the tadpoles (Horb et al., 2003). There is also evidence that the same construct can transdifferentiate the human liver cell line HepG2 into amylase or insulin expressing cells (Li et al., 2005).

1.1.4 Conceptual categorisation of metaplasia

The examples given above and the numerous homeotic transformations caused by mutations are clearly examples of metaplasia. However, I want to further clarify some terms usually found in the literature concerning metaplasia and transdifferentiation. Important concepts in understanding the switch of different cellular lineages are transdetermination, cell fusion and dedifferentiation.

1.1.4.1 Transdetermination

Transdetermination is defined as the switch of a specific precursor/stem cell lineage to another lineage (Tosh and Slack, 2002). The result is a change of

the original progenitor /stem cell fate that gives rise to descendants of the second lineage instead. This belongs to our other category of metaplasia but is not transdifferentiation. For example, imaginal discs transplanted between *Drosophila* larvae and adult female fly after many generations will sometimes change cell fate (Gehring, 1998). Imaginal discs which normally form legs will sometimes be transformed to become wings. There are even cases when bristles (small hair of the *Drosophila*) from a leg identity changes to one of an eye. Intriguingly, this is not a clonal event, but a process that will occur in groups of cells. This implies that transdetermination of the imaginal disc cells is not just a process of somatic mutation of the master regulatory genes (such as the Hox family) controlling the cell fates of the imaginal discs, rather, the process correlates with environmental cues during metamorphosis and also the extent of cell proliferation (many generations of transplantation). Signals such as *Wingless* (*wg,* the *Drosophila* Wnt) interacting with *Decapentaplegic* (*Dpp,* a member of the TGFβ family) in the leg disc, may account for the transdetermination of the dorsal leg cells to wing lineage (Maves and Schubiger, 1995; Maves and Schubiger, 1998).

1.1.4.2 Stem cells and metaplasia

The study of adult stem cells changing cell fates and give rise to descendants other than their own lineage is currently a very popular topic. Many scientists refer to this as transdifferentiation. However, considering our definition of transdifferentiation, which is conversion of cells from a differentiated state to another differentiated state, metaplasia concerning stem cells should be more appropriately considered as transdetermination (Fang et al., 2004). There are many examples of metaplastic cells where the source was considered to be

from adult progenitor/stem cells. For instance, cells derived from murine skeletal muscle have the potential to form haematopoietic lineages (Jackson et al., 1999), also neural stem cells possess the ability to form tissues from all the three germ layers in chimeric mice (Clarke et al., 2000). There are cases of haematopoietic stem cells (HSC) giving rise to neural cells (Eglitis and Mezey, 1997). Mesenchymal stem cells (MSC) and multipotent adult precursor cells can also form cell types of virtually all three layers (Krause et al., 2001; Jiang et al., 2002). HSC and MSC constitute what we know as bone marrow-derived cells (BMDC). It is proposed that highly plastic BMDCs have a role in maintenance and repairing of the non-hematopoietic tissue (Grove et al., 2004). The pluripotency of BMDC will be discussed in more detail in the next section.

1.1.4.3 Cell fusion

Bone marrow contains two populations of stem cells - (a) haematopoietic stem cell (HSC) which normally gives rise to all mature lineages of blood, and (b) mesenchymal stem cell (MSC) which can differentiate to bone, cartilage and fat. Bone marrow-derived cells (BMDC) are also capable of undergoing metaplasia (Pomerantz and Blau, 2004). Although recently, there are some incidences where direct conversion of BMDC to cells other than descendants of HSC and MSC lineages can occur (e.g. pancreatic endocrine cells derived from bone marrow without evidence of cell fusion (Ianus et al., 2003) and haematopoietic stem cells convert into liver cells without fusion (Jang et al., 2004)), most BMDC metaplasia appeared to be due to cell fusion. From the many examples of metaplasia involving BMDC changing phenotype to non-haematopoietic organs, there is now a growing body of evidence showing the ability of BMDC to form fusion heterokaryons with the target cells to rescue

the phenotype (Pomerantz and Blau, 2004). BMDC are known to be pluripotent and can directly switch to non-haematopoietic cell types. It is now recognised that BMDC possess the ability of cell-cell fusion with other cell types in the target tissues. Ultimately BMDC can repair the organ, albeit this happens very rarely. So far, examples of BMDC fused with liver, skeletal muscle, cardiac muscle and neurons have been found (Pomerantz and Blau, 2004). While there is evidence of metaplasia of BMDC changing into satellite cells in irradiated or exercise-induced muscle damage (transdetermination) (LaBarge and Blau, 2002), there is also evidence that formation of the new functional myofibres are fused mature myeloid cells with the injured muscle fibres (Corbel et al., 2003). However, one disadvantage of using muscle as a model of metaplasia is the difficulty of distinguishing between the mature multi-nucleated muscle fibre and the incorporated nuclei during the fusion process. A simple diagram of how BMDC may contribute in regenerative medicine is shown in Fig 1.3.

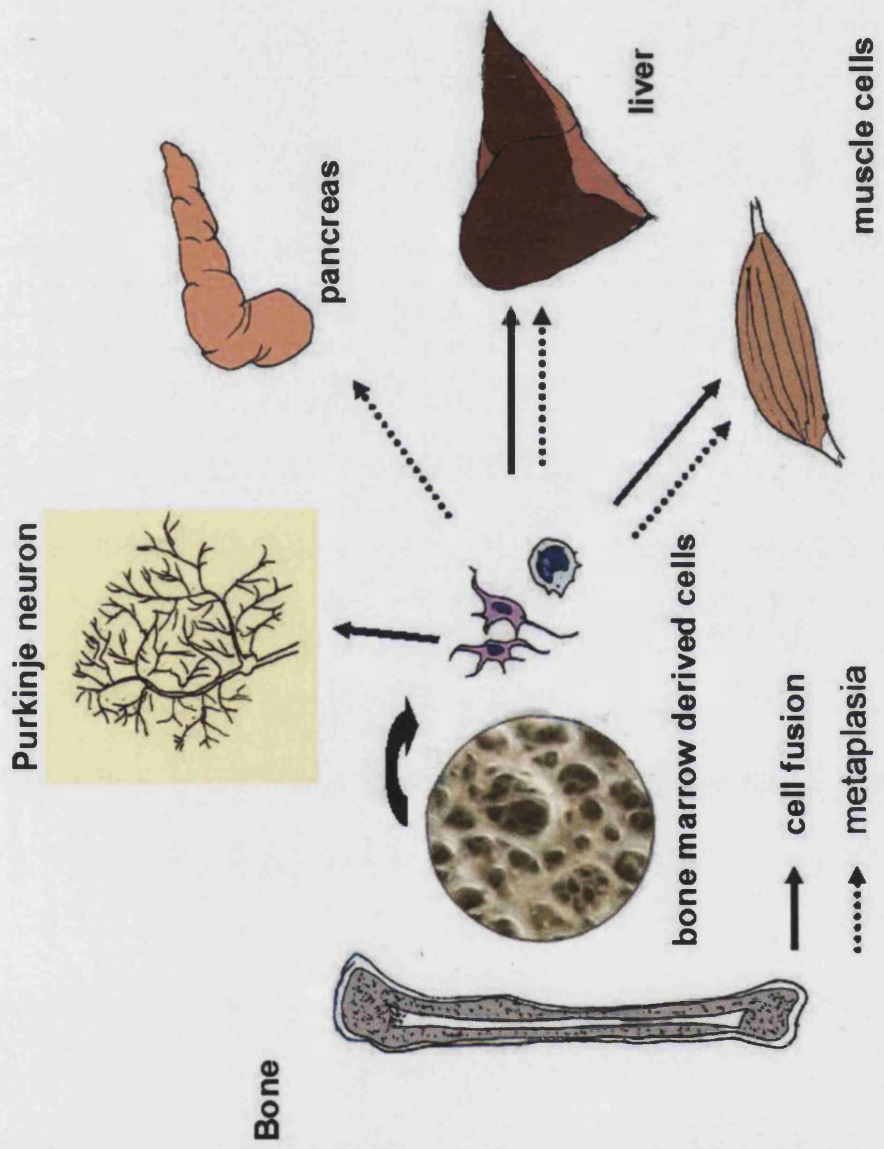

Purkinje neuron
pancreas
liver
muscle cells
Bone
bone marrow derived cells
cell fusion
metaplasia

Fig 1.3 Examples of cell fusion and direct transdifferentiation of BMDC in many tissue types.

Bone marrow-derived cells may represent a new avenue for regenerative medicine or repairing damaged tissue. There are now examples that BMDC could either incorporates (cell fusion) or direct conversion into the target tissues, found mostly after transplantation of BMDC into animals. Some examples are shown here, BMDC could be found fused to neurons, liver and (skeletal and cardiac) muscle cells, while there is also evidence showing direct conversion of BMDC to become pancreas, hepatocytes and muscle stem cells.

1.1.4.4 Cell lineage tracing

In order to look further into this subject, it is necessary to be able to trace the specific cell lineage of interest. Cre/lox recombinase is now one of the methods of choice for tracing cell lineages *in vivo* (Branda and Dymecki, 2004). For example, one can produce a bigenic animal by mating a transgenic mouse carrying a cell type-specific gene promoter driving Cre recombinase with another line carrying a LacZ reporter gene flanked by the loxP and stop codon that can be excised out when Cre recombinase is present (such as the R26R line). Following the excision event, any cell that has ever activated a specific promoter of interest will continue to express LacZ even after the cell stops using that specific promoter in question. This is a powerful method for tracing cell lineages as it is now possible to mark specific cell types which only transiently activates a specific promoter. Other cell lineage tracing methods utilise cytogenetic markers (such as tracing Y chromosome, when grafting male donor cells into female recipients) or simply lipophilic dyes (e.g. DiI) to label the cells of interest.

Using the Cre/lox system to detect cell fusion events, Alvarez-Dolado et al found evidence of BMDC fused with hepatocytes and cardiomyocytes *in vivo*, which raises the possibility of cell fusion as a mechanism for development and maintenance of tissue (Alvarez-Dolado et al., 2003). Also there is the case of BMDC incorporated into the fully differentiated, non-proliferative Purkinje neurons in both human (Weimann et al., 2003a) and mouse (Alvarez-Dolado et al., 2003; Weimann et al., 2003b). Neural stem cells were also found adopting embryonic stem cell characteristics after fusing with the co-cultured embryonic stem cell (Ying et al., 2002).

Evidence that transplanting HSC into fumarylacetoacetate hydroxylase

deficient ($Fah^{-/-}$) mice could rescue the liver function indicates that HSC have the potential of changing differentiation status and give rise to functioning hepatocytes (Lagasse et al., 2000). Further evidence indicates that the $Fah^{+/+}$ mice HSC that reconstitute the $Fah^{-/-}$ liver actually fuse to the existing hepatocytes (Wang et al., 2003; Vassilopoulos et al., 2003). Using a cell lineage tracing approach, the authors found that most of the Fah expressing cells still contain the recipient Y chromosome. The question is which cell type constituted the fused hepatocytes in the Fah deficient mouse model? They first ruled out the possibility that hepatocytes of $Fah^{+/+}$ mouse were fused with the $Fah^{-/-}$ hepatocytes by analyzing the phenotype and sex chromosome of the reconstituted hepatocytes from serial transplantation of $Fah^{+/+}$ female hepatocyte into male $Fah^{-/-}$ liver (Willenbring et al., 2004). Furthermore, using the same method, they established the cell types in the donor's HSC that is responsible for the fusion. When a specific subgroup of the HSC derivatives – the granulocyte macrophage progenitors (GMP, the colony forming units of progenitors for ganulocytes, macrophages and dendritic cells) and one of the further differentiated myelomonocytic lineage – macrophages are used to graft into the recipients, they fuse with the recipient hepatocytes and confer the long-term regenerative potential of the serial transplanted liver.

It is now important to investigate whether a metaplastic event is due to cell fusion rather than a direct switch from one cell type to another. To distinguish these possibilities, the chromosome numbers in the 'metaplastic' cells should be checked to see if it is a product of fused cells; and if possible, the cell lineage of the two cell types should be traced. Although tissue damage is thought to be an inducer for BMDC to fuse with the target cells, the precise inducing signals and the reason why only some tissue types could be fused

and others could not are still not clear (Alvarez-Dolado et al., 2003). Examples of signals to look for include IL-3 (which is necessary for fusion of the BMDC with embryonic stem cell *in vitro* (Terada et al., 2002)), or prostaglandin F2A and IL-4 (which induce myoblasts fusion with the existing myofibres (Horsley et al., 2003)).

There is evidence that fused cells can become mononucleated again either by nuclear fusion or supernumerary nucleus elimination (Alvarez-Dolado et al., 2003; Wang et al., 2003). Whether the fusion product, the stable heterokaryons, could also re-activate post-mitotic, terminally differentiated cell types to undergo cell division is currently in doubt but awaits intensive investigation. The fused BMDC-Fah$^{-/-}$ hepatocytes must undergo proliferation to produce a new functional liver and rescue the otherwise lethal condition. Despite the limitation of the low rate of cell fusion, these results show that cell fusion can possibly be considered as an alternative route for tissue repair, and the quest to have control over the process, i.e. induce its happening in specific cell types, and increase the possibilities, continues.

1.1.4.5 Dedifferentiaiton

During transdifferentiation, one important question to be addressed during the conversion of cellular phenotype from one to another is whether the cells undergo dedifferentiation. Dedifferentiation describes the process where by a differentiated cell first reverts to a common stem or progenitor cell type which then re-differentiates into another cell type. Understand the process of dedifferentiaiton will help us gain more knowledge about the normal differentiation process, as detailed understanding will be needed to establish the relationship between differentiated tissue and de-differentiated tissue.

During newt limb regeneration, the muscle and cartilage under the wound epithelium of the amputated limb dedifferentiates and re-enters the cell cycle, followed by regeneration of cells along several lineages including cartilage, muscle and interstitial fibroblast (Brockes, 1997; Tsonis, 2000; Brockes and Kumar, 2002; Nye et al., 2003). However, transdifferentiation does not necessary occur in all examples of amphibian regeneration (Gargioli and Slack, 2004).

It is interesting to note that in examples of BMDC conversion to muscle cells or hepatocytes, there are cases where the progenitor cells of skeletal muscle (satellite cells) and liver (oval cells) are found in the target organs originating from the myeloid origin (Petersen et al., 1999; LaBarge and Blau, 2002). These observations provide evidence of re-differentiation along the new tissue lineages when metaplasia occurs (Petersen et al., 1999).

1.1.5 Importance of understanding metaplasia

Metaplasia and transdifferentiation are important to study for several reasons.

(1) It is likely that between organs that undergo transdifferentiation, a single gene that acts as a differential switch during the course of development to form the otherwise different organ, and when that gene is activated, transdifferentiation occurs (see diagram in Fig 1.1). The study of transdifferentiation thus helps us understand more about the developmental relationship of the two organs that undergoes metaplasia or transdifferentiation (the two organs or cell types may be interrelated). In order to underpin the mechanism for metaplasia / transdifferentiation, we may also gain the serendipitous (or the must have) knowledge to reveal more of the molecular and cellular processes and mechanisms of

organogenesis.

(2) Metaplasia found in human is often accompanied by dysplasia and neoplasia, this is of clinical importance and the treatment of such tumours often has a bad prognosis. Understanding metaplasia will help us find methods to stop the frequently occurring abnormalities.

(3) Metaplasia acts as a mechanism for regenerative medicine, e.g. the application of adult transdifferentiated (stem) cell as a source for other tissue specific organs in need. Understanding the mechanism of transdifferentiation may help us apply the same technique in inducing (embryonic) stem cells to differentiate to the cell or tissue types we need.

We are also aware that there is a general distaste for the term "transdifferentiation" arising from the controversy over stem cell potency and careful usage of the term is necessary (Raff, 2003; Wells, 2002).

1.1.6 Metaplasia in the oesophagus

The oesophagus is an interesting organ for studying metaplasia and transdifferentiation. The reason is that three types of cell-type conversions occur in the oesophagus:

First, during embryonic development of the oesophagus, the epithelium of the mammalian oesophagus changes from simple columnar to the mature stratified squamous. It is currently not known whether there is a direct change in the epithelial cell type or whether there is an outgrowth of a specific progenitor cell either already present in the oesophagus or migrates from outside the tissue. The change in the oesophagus epithelium will be the main focus of the present study.

Second, the muscle layers of the oesophagus change from submucosal

mesenchymal cell type to more specified muscular cell type. More importantly, the appearance of a skeletal muscle phenotype that was found after the appearance of the smooth muscle cells indicates there is a change from smooth to skeletal muscle in at least some region of the oesophagus.

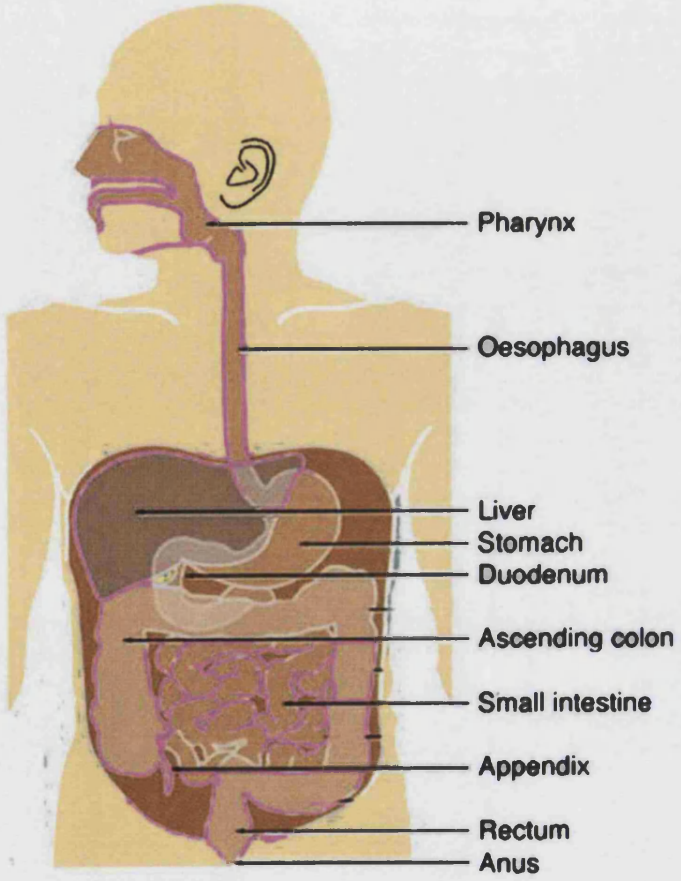
The third example relates to the clinical disorder - Barrett's metaplasia. Barrett's metaplasia (or sometimes referred to as Barrett's oesophagus) is an abnormality of the oesophageal epithelium tissue characterised by a change of the epithelium near the gastroesophageal junction from stratified squamous to intestinal columnar cell type (including Goblet cells). The change is thought to be due to repeated gastric reflux which presumably gives the symptom of heartburn. Understanding Barrett's metaplasia is important as it often predisposes to severe malignant adenocarcinoma. The prognosis of oesophageal adenocarcinoma is poor and the incidence is increasing in the Western world (Paulson and Reid, 2004). However, the molecular mechanism for Barrett's metaplasia to occur is still largely unknown.

1.2 Development of the mammalian oesophagus

1.2.1 Anatomical and cellular composition of the oesophagus

1.2.1.1 Basic anatomical description of the oesophagus development

In this thesis, the zoological convention is followed whereby "anterior" refers to the head end and "posterior" to the tail end. The oesophagus is derived from the anterior foregut endoderm. Just anterior to the oesophagus is a short segment of pharynx derived from the endoderm. More anterior than the pharynx is the oral cavity, which is derived partly from the endoderm and partly from the ectoderm (Waterman and Balian, 1980; Grapin-Botton and Melton, 2000). Along the anterior-posterior axis, at the ventral side of the oesophagus, are the endodermally-derived organs - trachea, bronchus, and lung. The thymus appendages and endocrine glands such as thyroid, and parathyroid also arise ventrally or laterally. Along the anterior-posterior axis, the oesophagus is followed by the gastroesophageal junction which connects to the stomach, there is then the liver / gallbladder and the pancreas, which is followed by small intestine, caecum, large intestine and anus (which is also partly endoderm and partly ectoderm) (Fig 1.4 Diagram of the human gut and a cross section of an oesophagus). The oesophagus functions mainly as a passage to allow masticated food to enter the gut for further digestion. Four distinct structural layers are designated to the whole adult gastrointestinal tract: mucosa, submucosa, muscularis propria and adventitia (serosa), in the order from the inner lumen to outer layer of the radiant axis of the gut (Young and Heath, 2001).


Pharynx
Oesophagus
Liver
Stomach
Duodenum
Ascending colon
Small intestine
Appendix
Rectum
Anus

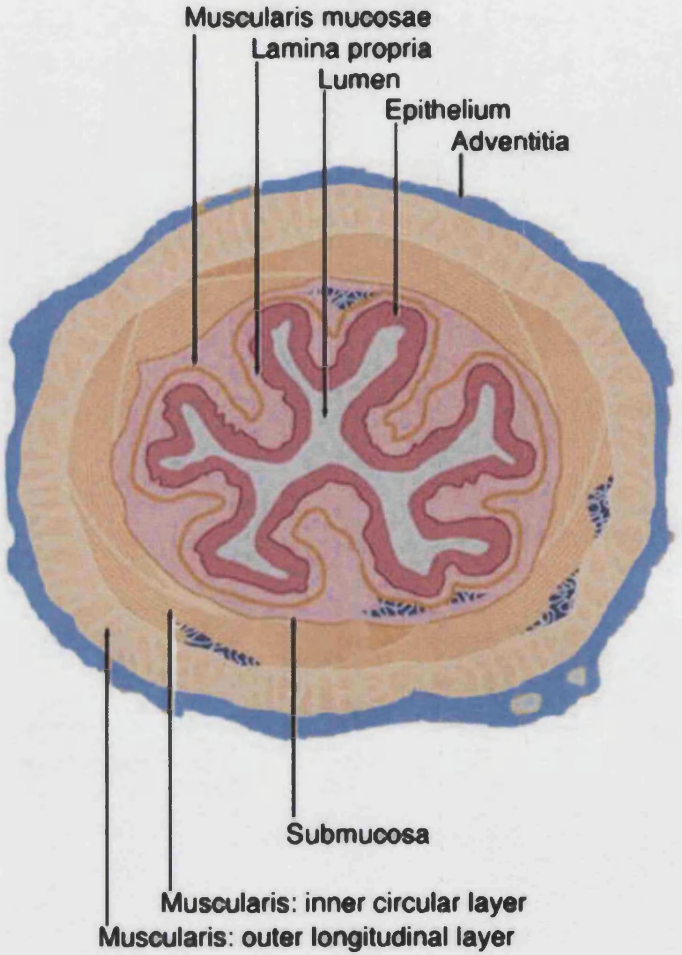

Muscularis mucosae
Lamina propria
Lumen
Epithelium
Adventitia
Submucosa
Muscularis: inner circular layer
Muscularis: outer longitudinal layer

Fig 1.4 Diagram of the human gut and cross section of an oesophagus.

A diagram showing the location and the anatomy of adult human oesophagus. The oesophagus is the tube-like structure linked from the pharynx to stomach. Mucosal epithelium is showed in dark pink on the right, submucosa mesenchyme in light pink, two muscle layers in yellow and adventitia in blue (Adapted from Young and Heath, 2001).

1.2.1.2 Initiation of the foregut endoderm

Much of the work on endoderm formation has been carried out in the chick. The definitive endoderm in chick is formed during gastrulation which emerged from the primitive streak (Slack, 2005). The gut tube itself is formed by the folding of the body away from the blastoderm, which can be thought of as a sort of evagination of all three germ layers (Fig 1.5). The head fold is formed by cells lifted off the blastoderm, the endoderm in this region is called foregut at this period. Later, a similar fold is formed at the posterior end forming the hindgut. Anterior intestinal portal (AIP) is the junction between the foregut and midgut, where the posterior intestinal portal (PIP) is the junction between the hindgut and the midgut. The AIP and PIP extend and meet at the midgut, where they close to form the gut tube, with a duct connecting the gut to the yolk sac. Non-endodermal tissue in the gut is derived from the splanchnic mesoderm and will be introduced later (section 1.2.1.4). Similarly, the mouse foregut is a single canal that forms after the AIP has folded into a tube-like structure. This folding occurs at the same time as somitogenesis begins (Lawson et al., 1986). At about E10, the foregut bifurcates into the trachea / lung buds and the oesophagus (Motoyama et al., 1998).

Signals like Sonic hedgehog (Shh) (see introduction of Shh signalling in section 1.2.4) and its downstream transcription factors *Gli2*, *Gli3*, are necessary for the formation of the oesophagus and in particular for bifurcation of the foregut (Motoyama et al., 1998). Another transcription factor *Nkx2.1*, is also important in the process of the separation of the foregut into the oesophagus and the trachea (Minoo et al., 1999). The phenotype of the *Nkx2.1* knockout mutant has the characteristic of the human newborn

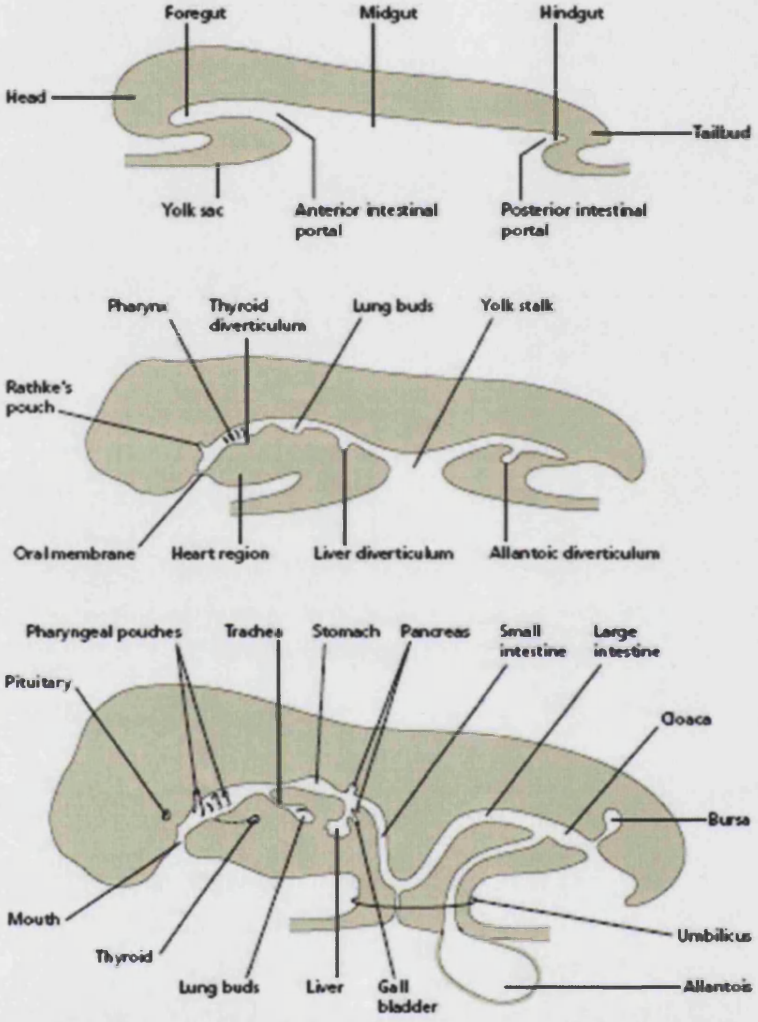

Foregut
Midgut
Hindgut
Head
Tailbud
Yolk sac
Anterior intestinal portal
Posterior intestinal portal
Pharynx
Thyroid diverticulum
Lung buds
Yolk stalk
Rathke's pouch
Oral membrane
Heart region
Liver diverticulum
Allantoic diverticulum
Pharyngeal pouches
Trachea
Stomach
Pancreas
Small intestine
Large intestine
Pituitary
Cloaca
Bursa
Mouth
Umbilicus
Thyroid
Lung buds
Liver
Gall bladder
Allantois

Fig 1.5 Diagram of the embryonic gut formation in the amniotes from a sagittal view (from Slack, 2005; with the permission of Slack J.M.W.).

abnormality - tracheoesophageal fistula (a fistula is an abnormal opening or passage between the trachea and oesophagus). In the forebrain (telencephalon), there is evidence that Shh signals to activate the transcription factors Gli1 and induces Nkx2.1 expression (Ruiz i Albata, 1998; Rallu et al., 2002). However, the role of Shh in activating Nkx2.1 is yet to be confirmed in the developing foregut.

1.2.1.3 Epithelium in the oesophagus - Columnar and stratified squamous cells

The structure and organisation of stratified squamous tissue has been well studied mostly in the epidermis. Fuchs and colleagues have shown that cytokeratin 14, an acidic intermediate filament cytoskeleton protein is a specific marker for the basal layer of the stratified squamous tissue, and is expressed in epidermis, cornea, vaginal epithelium and the oesophagus (Fuchs, 1988). Defects in this gene cause a human blistering skin disease called Epidermolysis Bullosa Simplex (EBS) (Coulombe and Fuchs, 1993). The formation and maintenance of stratified squamous tissue lies in the ability of cells to proliferate at the basal layer. Watt et al. described the stem cell and transit amplifying cell compartments in the adult epidermis (Jensen et al., 1999) and more recently, in the similar structure of the human oesophagus (Seery and Watt, 2000). Stem cells are presumed to reside in the inter-papillary region of the convoluted stratified squamous basal cell layer (Seery and Watt, 2000). Suprabasal cells of a stratified squamous tissue are found not to be proliferative. Differentiation of the suprabasal layers of stratified squamous tissue confer its capacity to withstand the environmental pressure it is subjected to and protect the organism from harm.

Previous studies in oesophageal development have shown that in the foetal

and neonatal mouse, the epithelial layers were 2-3 cells thick at E15.5 and a stratum corneum is found after birth (Raymond et al., 1991). Labelling of the proliferative cells in the epithelium of E18.5 mice showed localisation in the germinal layer (basal layer). There is a specific type of ciliated cell found in human, chick and mouse oesophagus during late embryonic development. This has protruding cilia and microvilli on the apical surface of the oesophageal epithelium that are visible under scanning electron microscope (S.E.M.), but these gradually disappear after birth. In human foetal oesophagus, at about 4 months of gestation, the columnar cells grow downward into the lamina propria, proliferate and then differentiate into the cardiac-type glands of the oesophagus (Johns, 1952). It has been speculated that the glandular tissue might be the source of Barrett's metaplasia (Jankowski et al., 1999).

Cytoskeletal proteins, involucrin, loricrin, filagrin and members of the small proline-rich protein family such as SPRR1 are expressed in keratinocytes of the cornified layers, but these are markers of many different differentiated stratified squamous tissues and are not restricted to the oesophagus (Steven and Steinert, 1994; Cabral et al., 2001). However, esophagin, one member of the *SPRR* family, is detected with an enhanced expression in the oesophagus (Smolinski et al., 2002). Esophagin is regulated by transcription factors such as C/EBPβ, Oct1 and Oct3, and may also be used as an oesophageal differentiation specific marker. Other intermediate filament markers that can be used to identify oesophagus include cytokeratin 4 and cytokeratin 13. These cytokeratins are expressed in the buccal, nasal, oesophageal, cornea epithelium, but usually not in the epidermis, trachea or lung (Pang et al., 1993; Waseem et al., 1998).

The main difference between a simple columnar and stratified squamous

epithelium is the multilayers of the stratified squamous epithelium. A columnar epithelial cell or basal cells in the stratified sqamous epithelium contains hemidesmosome (the cell junction between the basal lamina and basal cells), whereas cells in the stratified squamous suprabasal layers do not (Fuchs and Raghavan, 2002). Apart from the cytoskeletal proteins, the surface markers such as basal cell-specific, basal surface membrane marker β4-integrin, and polarity genes such as the apical PKCδ may also be used as specific markers to distiguish between the basal progenitor columnar and suprabasal layer stratified squamous cells (Lechler and Fuchs, 2005).

1.2.1.4 Non-epithelial cell types in the oesophagus: muscular and submucosal tissue

The outer coat of the gut is comprised of smooth muscle, connective tissue and blood vessel. This mesenchymal region of the gut is derived from the splanchnic mesoderm, the inner subdivision of the lateral plate following the opening of the coelomic cavity (Fig 1.6 (a)-(c))(Slack, 2005). The combination of the splanchnic mesoderm and the endoderm is called splanchnopleure.

The relationship between the prospective regions in the endoderm and those in the splanchnic mesoderm in the chick has been determined using cell labelling techniques (Matsushita S. et al., 2002). The prospective regions in the mesoderm tend to be arranged longitudinally and are arranged quite differently in comparison to the endoderm (Fig 1.6 (d)). For example, the oesophageal epithelial cells arises from endodermal cells dorsal to the 1st and 2nd somite, while the mesenchymal tissue which later surrounds the oesophagus tube comes from the splanchnic mesoderm that is lateral and positioned between the 1st and 7th somite. This shows that there is a

considerable relative movement of the two germ layers during the process of closure of the gut and closure of the ventral body wall. The fate map study shows that a particular region of endoderm experiences contact with different regions of the mesoderm as the gut tube formation process takes place, and the inductive interactions between mesoderm and endoderm constantly changes throughout gut development. The state of specification of the endoderm may change as it contacts different mesenchyme in the process.

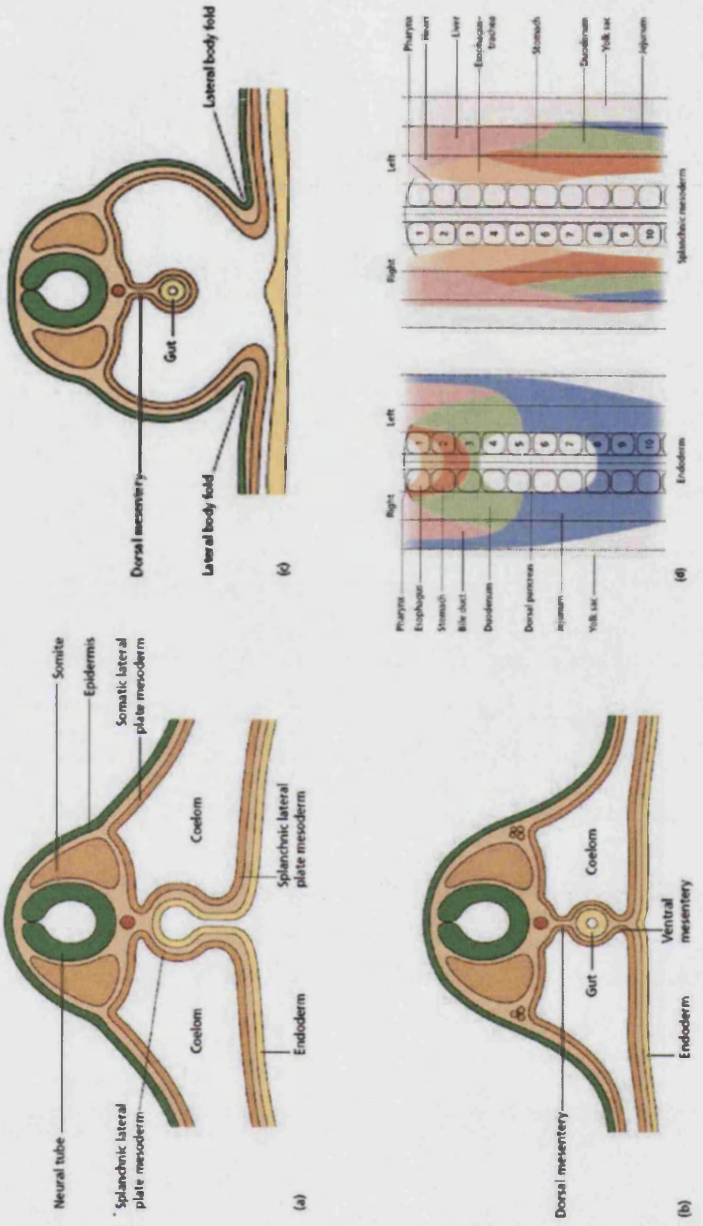

Somite
Epidermis
Somatic lateral plate mesoderm
Coelom
Splanchnic lateral plate mesoderm
Neural tube
Splanchnic lateral plate mesoderm
Coelom
Endoderm
(a)
Lateral body fold
Gut
Dorsal mesentery
Lateral body fold
(c)
Coelom
Ventral mesentery
Gut
Endoderm
Dorsal mesentery
(b)
Left
Right
Pharynx
Heart
Liver
Esophagus
Trachea
Stomach
Duodenum
Yolk sac
Jejunum
Splanchnic mesoderm
Left
Right
Pharynx
Esophagus
Stomach
Bile duct
Duodenum
Dorsal pancreas
Jejunum
Yolk sac
Endoderm
(d)

Fig 1.6 Endodermal and mesodermal cell origins in embryonic gut formation. (a)-(c) Cross-section views of the gut enclosure and ventral closure of the body in the chick embryo and (d) mismatch between the fates of endoderm and splanchnic mesoderm depicted at 1.5 days chick (from Slack, 2005; with the permission of Slack J.M.W.).

One form of metaplasia found in the oesophagus is the transdifferentiation of the muscle layers of the muscularis propria from smooth muscle to skeletal muscle. Samarasinghe showed that striated muscle is found at the rostral end of the mouse oesophagus, extending caudally with a mixture of both smooth and skeletal muscle, with smooth muscle alone being observed near the stomach (Samarasinghe, 1972). Patapoutian and colleagues demonstrated this is an example of transdifferentiation by showing skeletal and smooth muscle specific markers in the same muscle cell of an oesophageal tissue section (Patapoutian et al., 1995), although this interpretation is not universally accepted (Rishniw et al., 2003). It is unclear whether the smooth-to-skeletal transition is cell autonomous or occurs in response to external signals. But from the results of *Mammalian achaete-scute homolog 1* (*Mash1*$^{-/-}$) knockout mice, where no innervation of the enteric neurons from the neural crest can occur, the transdifferentiation of the muscle in oesophagus still continues, this suggests at least enteric neuron stimulation is not necessary for the muscle transdifferentiation (Sang et al., 1999).

1.2.2 The role of mesenchymal signals in endoderm development

Reciprocal signals between the endoderm and mesenchyme are known to be important for the specification of the whole gut (Grapin-Botton and Melton, 2000). Below are four examples of the signalling crosstalk between the endoderm and the mesenchyme in the gastrointestinal tract during embryonic development:

(a) Shh is ubiquitously expressed in the gut endoderm with the exception of the pancreatic region (Hebrok, 2003). Shh controls the bifurcation of the oesophagus and the trachea in the foregut region (Motoyama et al., 1998). A compound knockout mouse absent for the downstream transcription factors of Shh, which are *Gli2* and *Gli3*, lacks trachea, oesophagus and lungs. Also, repressive signals such as activinβB and fibroblast growth factor 2 (FGF2) from the neighbouring notochord control the non-expression of Shh in the region of endoderm that will form pancreas (Kim et al., 1997; Hebrok et al., 1998; Kim and Hebrok, 2001). When Shh is ectopically expressed (by the Pdx1 promoter) in the endodermal region of the pancreas (Apelqvist et al., 1997), the underlying mesenchyme will change to intestine-like smooth muscle and interstitial cells of Cajal of the intestine. Shh has been shown to induce *Hox* expression in the hindgut, but not in the foregut, for review see (Roberts, 2000). How the same signal - Shh induces different mesenchymal identity and organogenesis programmes along the forming gut is not clear.

(b) After hepatic specification at around E8-E8.5, the liver-specific genes *albumin* and *alpha-fetoprotein* are induced in the hepatic cells located in the foregut endoderm. Prior to morphological changes and establishment of other hepatic functions, the few liver marker-positive cells undergo cell

proliferation before morphogenetic movements towards a budding liver (Zaret, 1998). *Hlx*, a homeobox transcription factor, expressed in the mesenchyme of the septum transversum, is thought to be responsible for the secretion of the scatter factor / hepatocyte growth factor (HGF) (Hentsch et al., 1996). c-Met, receptor tyrosine kinase for HGF, is located on the epithelial hepatocyte membrane. When HGF binds to its receptor, c-Met is activated and stimulates the downstream activity of JNK (c-Jun N-terminal kinase) pathway. JNK in turn phosphorylates c-jun - one of the components of the transcription factor complex AP-1. The signal from the mesenchyme eventually leads to proliferation of the specified hepatocytes before budding morphogenesis occurs (Rodrigues et al., 1997; Auer et al., 1998). Hence there is a similar hypoplastic liver phenotype of mice homozygous mutated for *Hlx*, *HGF*, *c-met* and *c-jun* molecules, because the effect is probably on the same pathway (Hilberg et al., 1993; Schmidt et al., 1995; Bladt et al., 1995; Hentsch et al., 1996).

(c) The rat intestinal endodermal epithelium can be reprogrammed, to some extent, to a different tissue type by recombining with mesenchyme from another tissue (Duluc et al., 1994). However the reprogramming is restricted to particular cell types. E14 rat colonic epithelium re-combined with the small intestine mesenchyme was grafted under the skin of nude mice for 5 weeks to allow further differentiation. The reprogrammed epithelium formed small intestine-specific crypt-villi morphological structure and expressesed sucrase-isomaltase (SI) and lactase phlorizin hydrolase (LPH) - enzymes that are normally only expressed in the absorptive epithelium of the small intestine. However, using stomach or lung epithelium recombined with small intestine mesenchyme will not

change its original epithelial identity. In the case of the colon-to-small intestine epithelial conversion, the expression of caudal (posterior) related *Hox* genes shifted to the more cranial (anterior) expression pattern of the endoderm (Duluc et al., 1997).

(d) The chick non-glandular stomach (later to become gizzard) still retains a degree of plasticity as late as E9 and can be influenced by signals emanating from the mesenchyme, although cyto-differentiation has already begun. If the non-glandular endodermal epithelium is associated with glandular stomach mesenchyme, it can give rise to pepsinogen expressing glands (glandular stomach epithelium). If the stomach epithelium is combined with small intestine mesenchyme, it can even give rise to intestinal-type endocrine cells (Andrew and Rawdon, 1990; Mizuno and Yasugi, 1990).

Much is still not known regarding the signals that specify the oesophagus in the region of the foregut. In the current study, I focused on two important signalling pathways - the Wnt and Shh pathways. The function of Wnt and Shh in the gut and their relevance to the study is introduced below.

1.2.2.1 Wnt signalling in the gut

Wnt signalling is important in cell fate determination and cell proliferation (Logan and Nusse, 2004). A number of different Wnt isoforms are expressed in the developing gut (Lickert et al., 2001; Wang et al., 2001). At E12.5 and E16.5, Wnt5b and Wnt6 are expressed in the epithelium of the mouse oesophagus. Wnt4 is expressed weakly in the oesophagus. Wnt11 is expressed strongly at the gastroesophageal junction, but is in epithelial cells of the oesophagus and mesenchymal cells of the stomach. It is possible that Wnt signalling functions

in the development of the oesophagus. In mice that lack the frizzled-4 Wnt receptor, the oesophagus is distended and is dysfunctional, but the defect is probably due to failure of the oesophageal skeletal muscle formation (Wang et al., 2001). Lack of frizzled-4 is thought to affect the Wnt/Ca^{2+} pathway rather than the canonical pathway.

β-catenin is a pivotal gene in canonical Wnt signalling and serves as a multifunctional adaptor protein and a transcriptional cofactor (Nelson and Nusse, 2004). β-catenin can servie as a structural protein that forms a complex with α-catenin and p120 (or γ-catenin), linking actin to the cadherin family on the cell membrane (e.g. E-Cadherin in the epithelial cells). Normally, turnover of the β-catenin molecule is via a cytoplasmic degradation complex that contains axin, Adenomatous Polyposis Coli (APC), glycogen synthase kinase-3β (GSK-3β), and casein kinase 1 (CK1) which will phosphorylate β-catenin. Subsequently, phosphorylated β-catenin will be ubiquitinated and degraded in the proteasome. However, when cells are in contact with secreted Wnt, the Wnt receptor-frizzled and lipoprotein receptor-related protein 5 and 6 (LRP5/6) are activated. These receptors transduce a signal to several intracellular proteins that include Disheveled (Dsh), GSK-3β, Axin, APC, and the transcriptional regulator - β-catenin. Phosphorylation and degradation of β-catenin is then inhibited. β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus, where it binds to the transcription factor-LEF/TCF family leading to activation of many target genes, e.g. cyclin D1 (Shtutman et al., 1999) and c-myc (He et al., 1998).

1.2.2.2 Shh signalling in the gut

As mentioned earlier (sections 1.2.1.2 and 1.2.2), Shh signalling is important in

the bifurcation of the oesophagus and the trachea (Litingtung et al., 1998). In *Gli2*$^{-/-}$*Gli3*$^{-/-}$ knockout mice, the oesophagus and trachea form a single shortened tube and have a serious lung malformation (Motoyama et al., 1998). The role of hedgehog is not totally understood but it is known to be important in many processes of developmental patterning and maintenance of many adult tissues (Ingham and McMahon, 2001; Bijlsma et al., 2004). Hh signalling requires an autocatalytic cleavage process to becomes active, producing N-terminal (Hh-N) and C-terminal (Hh-C) products (Bijlsma et al., 2004). The active ligand is the Hh-N attached to lipophilic moieties. A cholesterol moiety is covalently attached to the C-terminal of Hh-N, which is so far the only known case of protein sterolation. Another modification is the covalent palmitoylation of the N-terminal of Hh-N, it is also the only known secreted protein to be palmitoylated. The sterolation is thought to affect the spatial distribution of Hh signalling, and palmitoylation is considered to enhance the ligand's potency. The Hedgehog receptor (Patched) negatively regulates another membrane protein - Smoothened. In the absence of Hedgehog, the downstream signal mediated by Smoothened is inhibited by an unknown inhibitory small molecule probably pumped out of the membrane from Patched. When Hedgehog is present and binds to Patched, Smoothened is relieved from the inhibition. When hedgehog signalling is active, the zinc finger transcription factor-*Cubitus interruptus (Ci)* in *Drosophila* (*Gli* family proteins in mammals) will then be released from the microtubule complex which includes the kinase Fused (Fu), kinesin motor protein Costal 2 (Cos2) and Suppressor of Fused (SUFU). The activated (intact) form of *Ci (Ci155) / Gli* will translocate into the nucleus and activate the downstream targets, such as *HNF3β*, *Hox* genes, *Bmp*s, etc (Ingham and McMahon, 2001). Shh was found to be expressed in the mouse

oesophageal epithelium until at least E15.5 (Litingtung et al., 1998).

1.2.3 The Hox clusters

An important process for anterior-posterior (AP) patterning in the animal kingdom is the function of homeobox genes – the *HOM-C* family of *Drosophila* or the *Hox* clusters in vertebrates (Kawazoe et al., 2002). The feature of *Hox* gene clusters is that there is a spatial and temporal colinearity expression pattern from the 3 to 5 along the chromosome which also corresponds to axial anterior-posterior positional information. The *Hox* clusters patterns the paraxial mesoderm (which later forms the vertebrae), neural tube, neural crest, hindbrain segments (the nervous system), branchial arches, and most likely, the gastrointestinal tract. This means that the sites and timing of expression of Hox genes along the anterior-posterior axis reflects its relative order of the genes on the chromosome. There are 4 clusters of the *Hox* genes in mouse – *Hoxa*, *Hoxb*, *Hoxc*, *Hoxd* - with each clusters containing 13 potential Hox gene sites, while none of the clusters contains all 13 genes (see Figure 1.5). *Hox* genes are expressed in both the endodermal and mesodermal region of the gut. Several studies have tried to demarcate the expression profile of the *Hox* clusters in the gut, and the results are summarised in Table 1.1. It may be possible that, with the combination of the expressing *Hox* code, we could specify each organ's identity along the AP axis of the gastrointestinal gut. We could also utilise the *Hox* gene expression pattern as markers to identify each specific endoderm organ.

One example of the importance of the *Hox* genes in oesophagus development is provided by *HoxC4*. The homozygous mutant of *HoxC4* in mice showed a partial or complete blockage of the oesophageal lumen in addition to the

vertebral defect which is from the second to the eleventh of the thoracic vertebra (t2 to t11), and an over-proliferation of the oesophageal epithelium (Boulet and Capecchi, 1996). The major reason for lumen obstruction is probably due to the disorganization of the esophageal musculature, which may cause the lack of the structural support to maintain the space for the lumen. It is not clear whether HoxC4 targets directly to the oesophageal muscle, or HoxC4 acts indirectly through patterning proper myoenteric neuron formation, so that lack of HoxC4 causes reduced myoenteric neuron stimulation to the muscle layers to function. Although *Hox* genes are also found in the foreguts of other animal models (such as *Quox-1* in quail (Xue et al., 1991)), and many genes of the *Hox* families are found spatially and temporarily regulated in the differentiated epidermis and hair follicle (Stelnicki et al., 1998; Reid and Gaunt, 2002), so far there is no evidence that any *Hox* gene is directly required for the initiation of the oesophageal (or the epidermal) stratification programme.

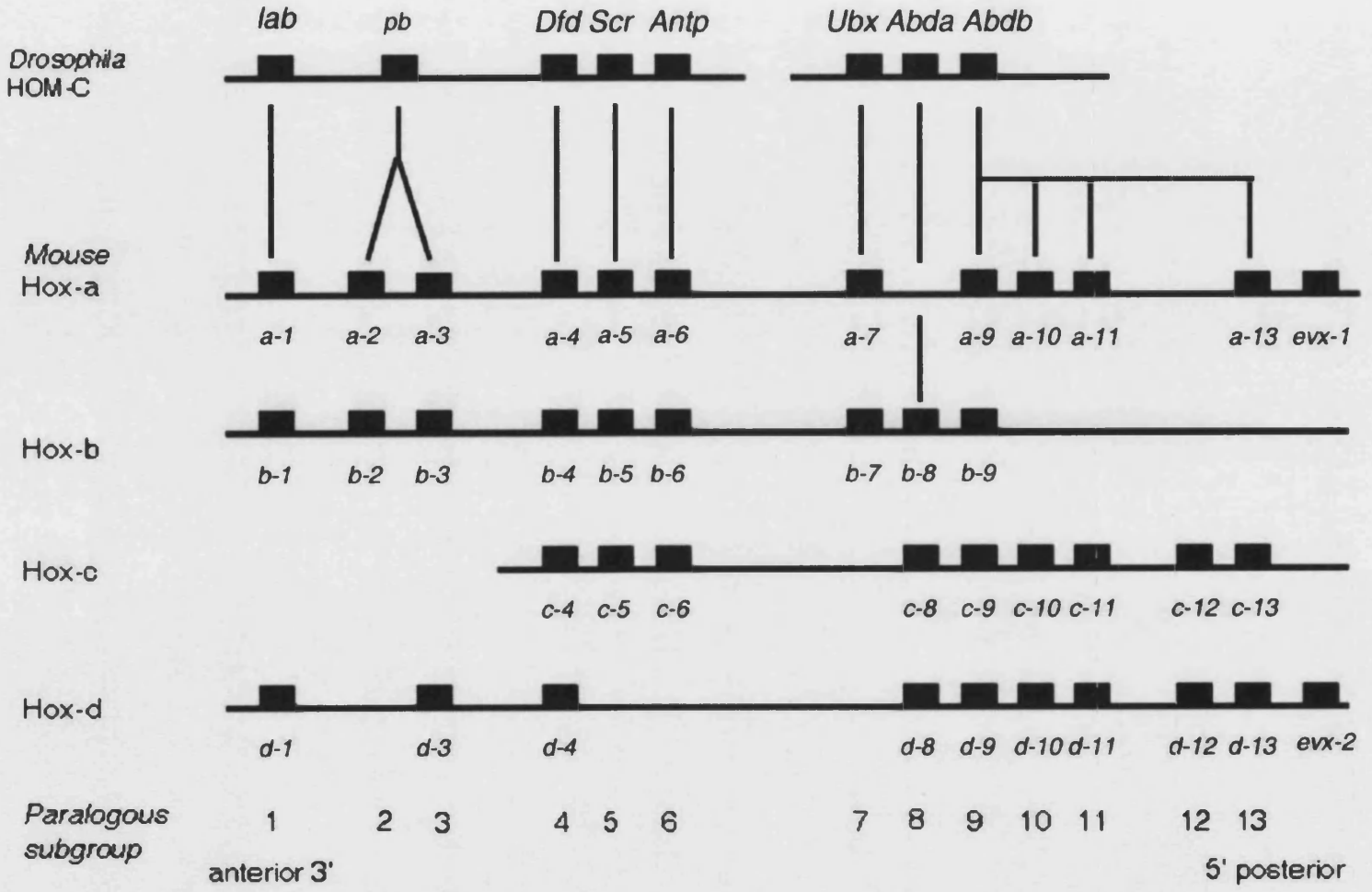

lab
pb
Dfd Scr Antp
Ubx Abda Abdb
Drosophila
HOM-C
Mouse
Hox-a
a-1 a-2 a-3 a-4 a-5 a-6 a-7 a-9 a-10 a-11 a-13 evx-1
Hox-b
b-1 b-2 b-3 b-4 b-5 b-6 b-7 b-8 b-9
Hox-c
c-4 c-5 c-6 c-8 c-9 c-10 c-11 c-12 c-13
Hox-d
d-1 d-3 d-4 d-8 d-9 d-10 d-11 d-12 d-13 evx-2
Paralogous
subgroup
1 2 3 4 5 6 7 8 9 10 11 12 13
anterior 3'
5' posterior

Fig 1.7 The phylogeny homology between the Drosophila *HOM-C* family and the four mouse Hox paralogous group. (adapted from Beck, 2002)

Table 1.1 Transcription Factors Expressed in Specific Organs (mostly Homeobox genes)

Oesophagus	HoxA2[1,2], HoxA3[1,2], HoxB4[1,2], HoxC4[3], HoxC5[3], Pax9[2], Six2[1], Sox2[1,2]
Stomach epithelium	HoxA2[1,2], HoxA3[1,2], HoxA5[3], HoxB4[1,2], HoxB5[3], HoxC5[1], HNF3γ[4], Six2[1], Sox2[1]
Stomach mesenchyme	Bapx1[1], Nkx2.5[1], HoxA4[3], HoxB4[3], HoxA5[3], HoxB5[3], HoxC5[3], HoxA6[3], HoxB6[3], HoxA7[3], HoxB7[3], HoxB8[3]
Intestine epithelium	Pdx1 (duodenum)[2], HoxA2[1,2], HoxC8[3], HoxA11[3], HoxD11[3], HoxA13 (colon)[1,2,3], HoxD13 (colon)[1,2,3], HoxC5[1,2], HoxC6[1,2], HoxB8[1,2], HoxB9[1,2], HoxC9[1,2], Cdx1[1,2], Cdx2[1,2], HNF3γ[4]
Intestine mesenchyme	HoxA4[3], HoxA5[3], HoxB6[3], HoxC6[3], HoxA7[3], HoxC8[1,3], HoxD8[3], HoxA9[3], HoxC9[1,3], HoxD9[3], HoxA10[1,3], HoxD10[1,3], HoxA11 (colon)[1,3], HoxD11 (colon)[1,3], HoxD12 (colon)[1,3], HoxA13 (colon)[1], HoxD13 (colon)[1], Bapx1 (hindgut)[1]

*: The expression profile of transcription factors in the gut of mice and chick was adapted and combined from several reviews.

(1) Roberts DJ. Molecular mechanisms of the development of the gastrointestinal tract. 2000. *Developmental Dynamics*, Vol 210, pp109-120.

(2) Grapin-Botton A and Melton DA. Endoderm development-from patterning to organogenesis. 2000. Trends in Genetics, Vol 16, pp124-130.

(3) Beck F, Tata F and Chawengsaksophak K. Homeobox genes and gut development. 2000. BioEssays, Vol 22, pp431-441.

(4) Kaestner K, Hiemisch H and Schutz G. Targeted disruption of the gene encoding hepatocyte nuclear factor 3 gamma results in reduced transcription of hepatocyte-specific genes. 1998. Mol. Cell Biol. Vol 18, pp4245-4251.

1.3 Development of the intestine

1.3.1 The anatomical structure, morphology and function of the small and large intestine

In the embryo, the small and large intestine originate from the hindgut. From anterior to posterior, the small intestine starts from the pyloric end of the stomach, including the duodenum, jejunum, and ileum. The caecum marks the separation of the small intestine and colon. The rectum is at the end of the gastrointestinal tract. In mouse intestine, cyto-differentiation starts from around E14 and is completed at 20 days after weaning. Roughly, intestinal development can be categorised into 3 phases.

(a) Villi formation at foetal stages - the differentiation along the crypt-villus axis is first seen between E17-18, when the endoderm is converted from a pseudo-stratified epithelium to an epithelial monolayer overlying nascent villi (Mathan et al., 1976).

(b) Crypt formation perinatally - crypts develop during the early postnatal period (days 1 - 5) from the flat inter-villus epithelium and mitotic cells segregate into these intervillus regions (Marshman et al., 2002).

(c) Maturation for digestive function after weaning. Differentiation of the epithelium also occurs along the anterior-posterior axis. The stem cells of the intestine are situated in the crypts, and can produce two daughter cells when undergoing cell division, one is a stem cell and one transit amplifying (TA) cell, the TA cells eventually differentiate into the four functional cell types of the intestine and will be introduced later in the section 1.3.4. The differentiated cells are situated above the stem cell position toward the tip of the villi except for the Paneth cell (see section 1.3.4). The most differentiated cells at the tip were constantly being shed off into the lumen.

Growth factors and hormones induce maturation of the intestine. These include glucocorticoids, which are important in maintaining intestine electro-neutral sodium absorption (Bastl et al., 1992), and are essential for intestinal cellular differentiation, such as the induction of the brush border enzymes – alkaline phosphatase, trehelase, and sucrase isomaltase. Glucocorticoid also induces premature accumulation of the goblet cells along the villi in the intestine if added in the culture from E17 *in vitro* (Beaulieu and Calvert, 1985). The glucocorticoid receptor, mineralcorticoid receptor and other non-classical corticosteroid receptors are expressed in the gut mucosa, and the 11-beta-hydroxysteroid dehydrogenase (11-β-HSD1 and 11-β-HSD2) enzymes are crucial for regulating and selecting between the ligands - glucocorticoid and aldosterone to function (Sheppard, 2002).

Another hormone stimulating the differentiation of the intestine is thyroid hormone. The circulating concentration of thyroid hormone is significantly increased during the second week after birth. Although there are 2 main subtypes of thyroid receptors (T3Rα and T3Rβ), thyroid hormone mainly acts through the T3Rα receptor to induce the morphological maturation of intestinal features (the villi are shorter in T3Rα mutants). Thyroid hormone is also important for proliferation of the epithelial cells, the expression of digestive enzymes and intestinal transcriptional factors such as Cdx1 and Cdx2 (Plateroti et al., 1999).

The function of the intestine is mainly digestion and absorption of food. After the initial physical breakdown of food from mastication, the salivary glands secrete amylase to digest carbohydrates; the food is swallowed and then enters the stomach through the oesophagus. In the stomach, the gastric glands process acidic hydrolysis of the food, a bolus is then formed and enters

the intestine. Digestion is carried out by enzymes whose secretion is controlled by various hormones. The bile and digestive enzymes are produced from the liver and pancreas, respectively. Another important function is the assimilation / absorption of the nutrients (including water) after their digestion, which is mainly done at the posterior end of the intestine (from the ileum to colon). The five cell types including the intestinal stem cells carrying out the functions of an adult intestine will be described in section 1.3.4.

1.3.2 Signalling pathways controlling the development of the intestine

Wnt signalling is a well studied pathway in the intestine. In the intestinal crypt, Wnt signalling suppresses cell cycle inhibitors like p21 and confers proliferative activity to the intestinal progenitor epithelial cells (van de Wetering et al., 2002). In canonical Wnt signalling, activated β-catenin accumulates in the nucleus, transactivating the *Tcf* target genes such as *c-Myc*, *cyclin D1*, *Tcf1*, *gastrin* and *PPARδ*, etc (van Noort and Clevers, 2002). When the Tcf-β-catenin pathway is disrupted, the progenitor cells arrest at G1 phase and differentiate.

Indian hedgehog (Ihh) expression at the villi restricts the Wnt signalling to the crypt (van den Brink et al., 2004). Ihh also regulates the transition of the epithelial precursor cells in the crypt into their differentiated derivatives. Wnt and Hh signalling was found to be down regulating each other in colonic epithelial renewal.

The Bmp pathway is also involved in the intricate regulation of intestinal cell fate. A recent publication of the mouse BMP receptor-BMPR1a conditional knockout model further revealed the subtle fine tuning of the intestinal stem cell (ISC) sensing autonomous and mesenchymal signals. Noggin, the BMP inhibitor was found secreted from the ISC (He et al., 2004). He et al.

established BMP as an inhibitory signal for proliferation of the intestinal stem cell. Bmp will block the Akt pathway by activating PTEN. When BMP signalling is inactivated by Noggin (secreted by the ISC), Akt signalling becomes activated and subsequently activates Wnt signalling. Accumulation of β-catenin in the nucleus in turn stimulates the proliferation of the stem cells in the crypt. Interestingly, Fkh6 (a transcription factor of the forkhead family containing the winged helix domain) is expressed in the mesenchyme adjacent to the intestinal epithelium and regulates Bmp2 and Bmp4 secretion in the intestine. The knockout phenotype shows that it is also a negative regulator of the crypt cell proliferation (Kaestner et al., 1997). Another transcription factor, Nkx2.3 is also expressed in the intestinal mesenchyme, the homozygote mutant mice produced the same phenotype, which is the expansion of the crypt cell compartment, and decreased expression of BMP2 and BMP4 (Pabst et al., 1999).

Ectopic expression of a secreted Wnt inhibitor - Dickkopf1 (Dkk1) suppresses intestinal cell proliferation and subsequently causes loss of intestinal crypts (Pinto et al., 2003). However, the secretory cell lineages in the intestine are also largely absent in this inhibitorory Wnt signalling mutant mouse model, showing that the Wnt pathway is also involved in lineage determination.

In conclusion, signalling pathways such as Bmp and the canonical Wnt inhibitor-Dkk1 are negative signals secreted by neighbouring cells of the intestinal stem cell to supress ISC proliferation. Noggin, the suppresser of the Bmp pathway is secreted by ISC to counteract these negative signals. The different signalling pathways are found to act at different developmental stages for the maintenance of ISC and regulation of differentiation along the intestinal cell lineages. Whether these signalling pathways themselves regulate one

another is worth pursuing.

1.3.3 Genes and Cell types

Transcription factors such as Tcf4, Cdx1, Fkh6, HNF3/HFH11 and Nkx2.3 are found localised at the crypt compartment and are important for maintaining the intestinal stem cell identity and function (Clatworthy and Subramanian, 2001). Activating the Wnt - β-catenin complex will induce Cdx1 expression in mouse ES cells and rat embryonic endoderm, and so link the Wnt signalling pathway to Cdx1 function in the crypt (Lickert et al., 2000).

Mice in which one allele of the *Cdx2* gene had been inactivated by homologous recombination, develop multiple intestinal polyp-like lesions that do not express Cdx2 but contain areas of metaplasia in the form of keratinizing stratified squamous epithelium - the hallmark of oesophagus epithelium (Beck et al., 1999). $Cdx2^{+/-}$ mice showed "intercalary regeneration", in which small intestine, stomach and oesophagus tissue type cells form foci in the $Cdx2^{+/-}$ mice proximal colon polyps. Metaplastic epithelium were found to "fill in" the polyps following the AP axis sequence of the gut (colonic -> small intestinal -> gastric -> oesophageal, from peripheral to centre of the polyp). The expression of Cdx2 was totally lost from both alleles in gastric and oesophageal regenerative tissues in the centre of the polyps of the heterozygous mice. Intercalary regeneration implicates *Cdx2* as a candidate for determining the caudal (intestinal) patterning of the gut. Without the gene, intestine reverts to a default state of anterior tissue. This is a fine example of homeotic transformation in the mammalian gut. Cdx2 has also a role in regulating progenitor cell growth which when disrupted, caused tumour formation (Chawengsaksophak et al., 1997).

There is a functional difference in the intestine at different regions along the AP axis of the gut. Some genes may play a role in controlling the morphological and biochemical differences from the duodenum to the colon (Sweetser et al., 1988). Genes such as different types of *laminins*, *Cdx1* and *Cdx2*, have different expression levels along this anterior-posterior axis, showing expression gradients from the anterior to posterior of the small and large intestine. There might also be a correlation between the expression of different laminins (via induction of Cdx2) and the differentiation status in the intestine (Kedinger et al., 1998).

Another complementary gene expression pattern is in the crypt-villus axis. For example, integrin subunit α2 is expressed in the crypt and α3 subunit in the villi (Beaulieu, 1992). Cdx1 is located in the crypt region while Cdx2 is expressed in the more differentiated villous enterocytes (Clatworthy and Subramanian, 2001). This expression gradient pattern may provide a molecular basis for crypt-villus cellular differentiation.

1.3.4 Markers for different intestinal cell types

Intestinal cells types can at be divided into: common stem cell, absorptive enterocytes, mucin secretive cells, Paneth cells and enteroendocrine cells.

Results deduced from cell kinetic studies show intestinal stem cells are located about four cells up from the bottom of the crypt (Potten and Morris, 1988). Markers for the intestinal stem cell include *Musashi-1 (Msi1)*, an RNA binding protein and a mammalian neural stem cell marker that can induce a transcription repressor - *Hairy and Enhancer of Split* homologue (*Hes1*) (Sakakibara et al., 1996; Potten et al., 2003). Hes-1 is essential for neural stem cell renewal and suppression of neural stem cell differentiation (Nakamura et

al., 2000). Msi-1 and Hes-1 are found co-expressed in cells just above the Paneth cells (Kayahara et al., 2003), and can be used as intestinal stem cell markers.

The four differentiated cell types of adult intestine are:

1. Absorptive cells. These are the most abundant intestinal cell type. In the small intestine they are termed "enterocyte" and in the large intestine "colonocyte". Absorptive cells are polarised cells specialised for absorption by the presence of microvilli at the apical membrane. Villin is an actin bundling protein expressed on the apical brush border of absorptive cells. Although it is expressed in every cells of the crypt-villi axis, there is a gradient of villin expression with more at the villi and less at the crypt (Madison et al., 2002). Markers such as intestinal specific alkaline phosphatase (which is expressed embryologically)(Alpers et al., 1994), lactase phlorizin hydrolase (LPH) and sucrase isomaltase (SI) (usually expressed after weaning)(Traber and Silberg, 1996), are known as brush border enzymes. These enzymes are important for the digestive function of the absorptive cells. Cdx2, Pdx1, gut-enriched Krüppel-like factor, members of the *HNF1* family and the *GATA* family as well as glucocorticoid and thyroid hormones are known to regulate the expression of the enzymes mentioned above. The cell fate of the absorptive intestinal epithelia is probably determined via some intestinal progenitor cells that start expressing Hes-1, a transcription factor downstream of Notch signalling (Jensen et al., 2000). Hes-1 in turn suppresses the expression of Math-1 in those cells. Math1 is a downstream transcription factor of Delta receptor and is important in determining secretive cell fate in the intestine (Yang et al., 2001). The sequential activation of another transcription factor E74-like factor 3 (Elf-3), combining Hes-1 or Math-1 eventually leads to different lineage determination

in the intestine (Ng et al., 2002; Sancho et al., 2003).

2. Mucous-secretive (Goblet) cells are dispersed throughout the intestinal epithelium and secrete mucous to (a) lubricate the intestinal contents and (b) trap and expel unwanted micro-organisms. Glycoproteins – the rich cellular content of a goblet cell allows several histochemical techniques to be applied to visualise the presence of this cell type. Periodic Acid Schiff (PAS) and acidic alcian blue (pH 2.5) both stain the intestinal mucopolysaccharides revealing conspicuously stained mucigen of Goblet cells and are reliable markers for showing the goblet phenotype and morphology. Muc2 is a gel-forming secreted mucin expressed only in Goblet cells of the small and large intestine, and could be used for an intestinal specific marker (Corfield et al., 2000). The Delta-Notch pathway is important in specifying intestinal lineage cell fate. Math1, which is activated in the Delta ligand expressing cells (neighbouring cells of the Notch activated cells), is a transcription factor involved in committing cells to become the three secretory cell lineages - Goblet, Paneth, and enteroendocrine cells (Yang et al., 2001). A zinc finger transcription factor, *gut-enriched Kruppel-like factor-4* (*GKlf-4*) is important in Goblet cell maturation. GKlf-4 knockout mice showed a dramatic reduction in the number of Goblet cells, and patchy expression of Muc2 along the crypt of the colon (Katz et al., 2002). The Goblet cell lineage determination is considered to be controlled by the sequential expression of transcription factors Math-1, Elf-3 and Klf4 (Sancho et al., 2003).

3. Paneth cells are exclusively found at the crypt base of the small intestine and the ascending colon. The cells contain large apical secretory granules and express various proteins including lysozyme and anti-bacterial peptides called defensins or cryptdins. Metalloproteinase matrilysin (MMP-7)-dependent

procryptdin activation *in vivo* provides mouse Paneth cells with functional peptides for apical secretion into the small intestine lumen (Wilson et al., 1999; Ayabe et al., 2002). *Math 1* is important for the determination of the secretory cell lineages in the intestine including Paneth cells. It is worth noting that the nuclear form β-catenin is also found in the Paneth cells (van de Wetering et al., 2002).

4. Enteroendocrine cells. There are at least 10 different enteroendocrine cell types (Schonhoff et al., 2004). Markers for enteroendocrine cells are such as chromagranin A, synaptophysin, cholecystokinin A. Some types of the enteroendocrine cells can also be found in stomach, pancreas and the pituitary gland. Enteroendocrine cells function not only in embryonic development, but also during the constant renewal of gut epithelia in the adult. They are believed to be formed by regulation of the Notch signalling pathway. Notch is inactive in endocrine precursor cells, allowing the expression of the proendocrine basic helix-loop-helix proteins Math1 and neurogenin3 (Ngn3). Math1 is the first transcription factor involved in endocrine specification, committing cells to become one of three secretory lineages - Goblet, Paneth, and enteroendocrine (Yang et al., 2001). Neurogenin3 appears to be a downstream target that is essential for endocrine cell differentiation (Lee et al., 2002). The transcription factors Pax4, Pax6, BETA2/NeuroD, and Pdx1 have all been implicated in enteroendocrine differentiation (Schonhoff et al., 2004). BETA2/NeuroD coordinates *secretin* gene expression in S-type enteroendocrine cells with cell cycle arrest as cells terminally differentiate. Peptide hormones such as GLP-1, GLP-2 are secreted from the enteroendocrine cells in response to nutrient ingestion (Baggio and Drucker, 2004). GLP-1 enhances glucose-stimulated insulin secretion and inhibits glucagon secretion, gastric emptying and feeding.

GLP-1 also has proliferative, neogenic and anti-apoptotic effects on pancreatic beta-cells (Baggio and Drucker, 2004). GLP-2 is an intestinal trophic peptide that stimulates cell proliferation and inhibits apoptosis in the intestinal crypt compartment. GLP-2 also regulates intestinal glucose transport, food intake, gastric acid secretion and stomach emptying, and can improve the intestinal barrier protective function formed by the epithelial tight-junction (Baggio and Drucker, 2004).

1.4 Barrett's metaplasia

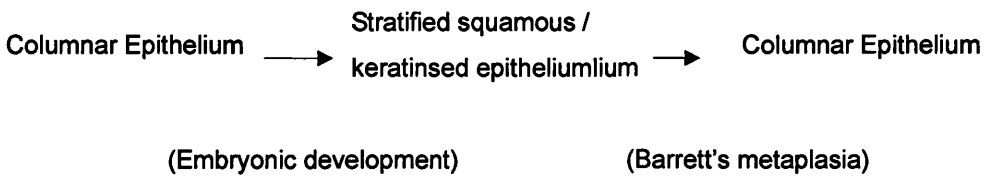
In the adult, the epithelia lining the gut are columnar except in the oesophagus where it is stratified squamous. A pathological change in oesophageal epithelia is observed in Barrett's metaplasia (BM). BM is characterised by a mucin-secreting simple columnar epithelium that forms glandular invaginations in the oesophageal mucosa, localising near the gastro-oesophageal junction (Fig 1.8). This is known as intestinal metaplasia of the oesophagus. Nowadays, the criteria for diagnosis of Barrett's metaplasia include the appearance of intestinal mucous secreting Goblet cells in the tissue biopsies (reviewed in Jankowski et al., 2000).

The main cause of Barrett's metaplasia is thought to be the reflux of bile acids and digestive enzymes from the stomach and intestine to the oesophageal mucosa and is often referred to as gastro-oesophageal reflux disease (GORD) (Jankowski et al., 2000). This is now very common in Western countries, where many patients complained about heartburn, a clinical symptom of BM (see section 1.1.6). 10% of adults screened by gastroenterologists have endoscopic evidence of oesophagitis due to GORD, and 10% of these people will progress to Barrett's metaplasia (Cameron et al., 1990). Oesophageal metaplasia is thought to be a strong prognostic indicator of adenocarcinoma of the oesophagus (the prevalence of oesophageal adenocarcinoma is increasing by 0.2~2% each year over the last decade)(Cameron et al., 1990). Once oesophageal adenocarcinoma is diagnosed, despite chemotherapy and surgery, patients have a median survival time of less than 1 year, and fewer than 10% of patients survive for more than 5 years (Cameron et al., 1990).

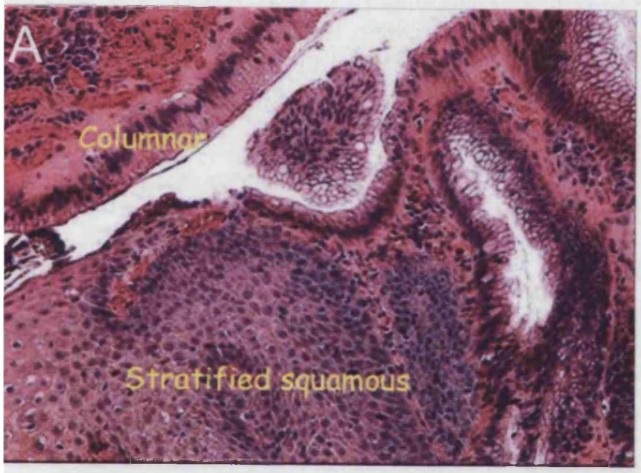
The current favoured theory of how Barrett's metaplasia arises is that over many years, repeated damage to stem cells located in the oesophageal

epithelia causes some of the renewing epithelial cells to change to intestinal-like epithelial cells (see diagram below for an explanation of the epithelial changes). It is still not clear whether it is the stem cell of the squamous tissue, or the oesophageal gland that undergoes metaplasia (Jankowski et al., 1999).

The epithelial changes of the oesophagus:



By using animal models and by monitoring the human oesophageal pH and bilirubin level, many investigators have tried to understand the role of acid and duodenal-gastric reflux in oesophageal mucosal injury. Bilirubin is a reddish-yellow bile pigment that is an intermediate product of the breakdown of haemoglobin in liver (Vaezi et al., 1995). There are two phases of the effects of bile and acid. At pH 2, HCl with gastric digestive enzyme pepsin and conjugated bile taurocholate, taurodeoxycholate and lysolecithin cause oesophageal mucous damage, while at neutral pH, unconjugated bile acid cholic acid with the digestive enzyme trypsin cause increased mucosal disruption (Vaezi et al., 1995). These results suggest synergistic damaging effects for both bile and acid in oesophageal mucosal injury.


A
Columnar
Stratified squamous

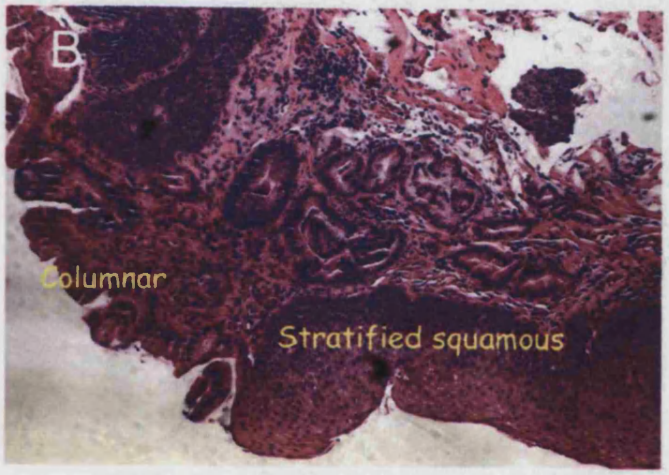

B
Columnar
Stratified squamous

Fig 1.8 Two clinical samples of Barrett's oesophagus.

Haematoxylin and eosin staining of two Barrett's metaplasia biopsy samples from the oesophagus of patients is shown. Note that the metaplastic glandular tissue is found on the epithelium layer near the lumen and also underneath the submucosal region of the stratified squamous epithelium. The images were 400x in A and 200x in B (Courtesy of Dr. Leigh Biddlestone, RUH, Bath).

1.5 Objectives of the current work

My main goal is to determine the mechanism for the development of the oesophageal epithelium, i.e. after the septation between the oesophagus and the trachea/lung buds, what makes the oesophagus develop from a simple columnar sheet of epithelium to a fully differentiated, cornified stratified squamous epithelium?

I start by describing the oesophageal muscular and epithelial morphology during mouse embryonic development and the production of a mouse *in vitro* culture system that mimics the normal development. The advantage of using mouse as an animal model is because it is genetically well defined (the genomic sequence of *Mus musculas* is completed), and the physiology and the genetics are evolutionary closer to human than other well studied model systems such as the *Drosophila* and *Xenopus*.

Next, I will demonstrate the cellular basis of the epithelial switch and answer the question of whether the columnar-to-stratified squamous is a direct conversion, or is an outgrowth of the stratified squamous progenitor cells. I will also try to answer whether cell death and cell proliferation is necessary during this phenotype switch.

I will then focus on the molecular mechanisms that control this event and consider whther the transcription factors controlling the initiation of the stratified squamous tissue in the oesophagus are similar to those in the epidermis. I also described perturbation of several signalling pathways to see if the process is regulated by these signals.

The last objective is to understand the molecular mechanisms of how the fully differentiated stratified squamous tissue will convert to a columnar intestinal phenotype, ectopically found in the Barrett's oesophagus. Our findings on how

stratified squamous epithelia formed from simple columnar cells in the oesophagus may be applicable to patients with Barrett's oesophagus and help to reverse its progress.

Chapter 2 Materials and Methods

2.1 Isolation and culture of embryonic oesophagus, trachea, lung buds, stomach and intestine

Female CD1 outbred albino mice (from Charles River) were mated overnight and the following morning was designated E0.5 if the presence of vaginal plugs was observed. Pregnant animals were killed by cervical dislocation and the uteri dissected out in ice-cold sterile phosphate buffered saline A (PBSA). E11.5, E13.5, E15.5 or E17.5 embryos were removed from the deciduas, transferred to ice-cold Minimum Essential Medium (MEM) with Hank's salts, 10% FBS and 50 µg/ml gentamycin and the gut (from pharynx to the intestine) was dissected free using a microscapel and fine forceps under a Leica dissecting microscope with indirect light source. The oesophagus was removed from its position rostral to the stomach and was separated from the trachea. Oesophagi were collected in ice-cold MEM / Hank's medium.

For organ culture, the oesophagus was placed on a coverslip subbed and coated with fibronectin. To sub, the coverslips were immersed sequentially in hot soapy water, reverse osmosis (RO) water and 95% ethanol / 0.1% acetic acid. The coverslips were allowed to air dry and then dipped for 10 minutes in 2% 3-aminopropyltriethoxysilane (APTES, Sigma) in the fume cupboard. Afterwards, the rack was dipped in two separate containers of acetone before washing in RO water. The coverslips were then dried at 37°C or heat sterilised overnight at 180°C. To coat with fibronectin, individual coverslips were placed in sterile 35 mm plastic dishes and then 40 µl of 50 µg/ml bovine fibronectin (dissolved in water (Life Technologies) was placed in the centre of the coverslip. Initially, a plastic ring of 5 mm internal diameter was placed over the

fibronectin-coated area and media (Basal Medium Eagle (BME) medium with Earle's salts, 10% foetal bovine serum, 2 mM glutamine (all from Life Technologies) and 50 µg/ml gentamycin (Gibco/Invitrogen) was pipetted dropwise into the cloning ring and thereafter into the rest of the dish up to 2.5 ml. The oesophagus bud was then dropped onto the centre. 24 hrs later, the cloning ring was removed and the media decanted and replaced with 2 ml. The cultures were grown in 37°C, 95% air/ 5% CO_2 humidified incubator for up to 20 days. The medium was changed every 2 days. Trachea, lung buds, stomach and intestinal cultures were performed under the same condition.

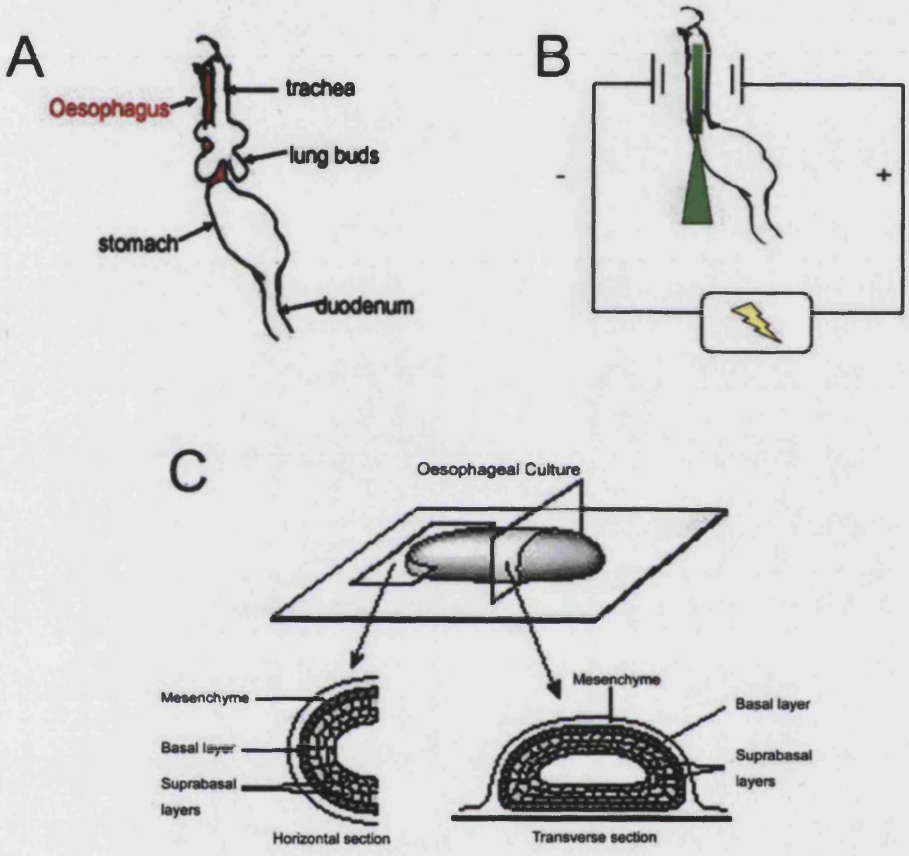

A
Oesophagus
trachea
lung buds
stomach
duodenum
B
-
+
C
Oesophageal Culture
Mesenchyme
Basal layer
Suprabasal layers
Horizontal section
Mesenchyme
Basal layer
Suprabasal layers
Transverse section

Fig 2.1 Diagram of the dissected E11.5 embryonic oesophagus.

A schematic diagram shown in A is the gut region of an E11.5 mouse, the oesophagus is marked in red (A). Note the oesophagus is at the ventral side to the trachea and lung bud. A simplified diagram showed in B is the electroporation technique used for delivering genes into the epithelium of the oesophagus, the approach can be used to deliver genes into other organs such as the stomach, intestine, trachea and lung buds. The views shown in C is a diagram of how the horizontal confocal pictures of an oesophagus culture looks. Mesenchyme is on the outside then the epithelial basal layer then several layers of suprabasal cells.

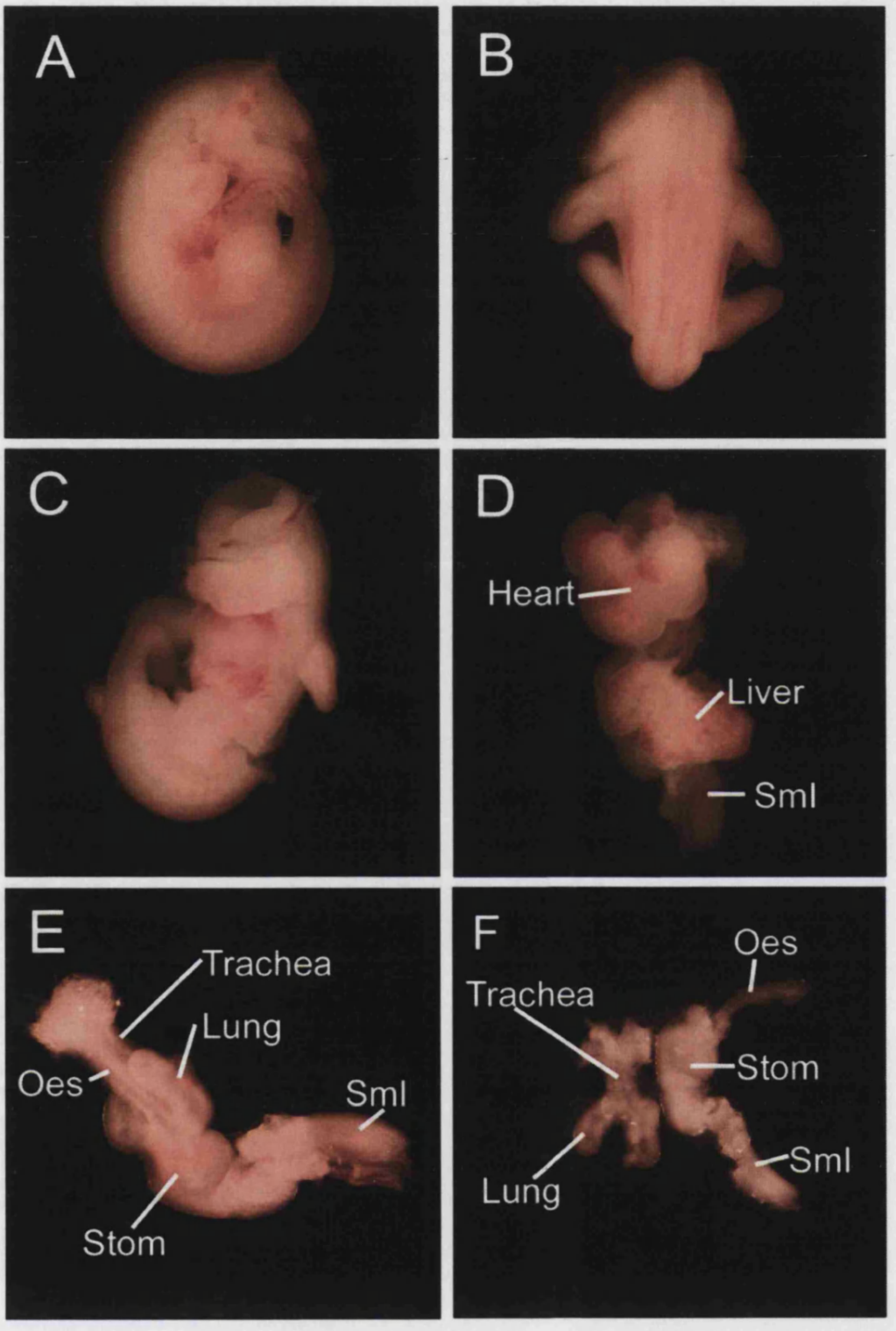

A
B
C
D
Heart
Liver
Sml
E
Trachea
Lung
Oes
Sml
Stom
F
Oes
Trachea
Stom
Sml
Lung

Fig 2.2 Stepwise illustration of the dissection of the mouse E11.5 oesophagus (and trachea, lung, stomach, intestine).

Photos of the lateral (A), dorsal (B) and ventral (C) views of an E11.5 embryo are shown. Head of the embryo has been removed. A ventral view of the internal organs after further removal of the integument and the outer part of the body is shown in D. The view of the gut region is blocked by heart and liver located ventrally (D). The oesophagus, trachea and lung buds, stomach and small intestine are shown in E after removal of heart and liver. The oesophagus, stomach and small intestine are shown in F after removal of the trachea and lung buds. The magnification of A, B and C is 50x, F is 100x and D, E is 200x.

2.2 Cell lines, transfection and culture conditions

All cell lines were purchased from European Collection of Cell Cultures (ECACC, Porton Down, U.K.) The culturing conditions of each cell line are as follows. KYSE30 was cultured in 50% RPMI-1640 and 50% F-12 HAM Nutrient medium (Sigma) supplemented with 2% FBS, 2 mM L-Glutamine, 0.5 U/ml of penicillin and 500 ng/ml of streptomycin (Life Technologies). OE33 was cultured in RPMI-1640 medium supplemented with 10% FBS, L-Glu and penicillin/streptomycin. Caco-2 was cultured in MEM medium (Sigma) supplemented with 10% FBS, 1% Non-essential amino acids (Life Technologies), L-Glu and penicillin/streptomycin. NIH3T3 4.2 was cultured in DMEM medium (Sigma) supplemented with 10% FBS, L-Glu and penicillin streptomycin. HepG2 was cultured in DMEM medium (Sigma) supplemented with 10% FBS, 1% Non-essential amino acids (Life Technologies), L-Glu and penicillin/streptomycin. Cells were cultured in 37°C, 95% air / 5% CO_2 humidified incubator and the medium was changed every 2 days; until 90% confluent (usually between 4 ~ 6 days) and subcultured at a 1:8 ~ 1:12 ratio. For transient transfection, generally, 1 μg of DNA was mixed with 3 μl of GeneJuice (Novagen) for 15 min in room temperature in 100 μl of serum free RPMI medium, and added to ~50% confluent cells for 24 hrs.

5-azacytidine (5-AzaC) 1 - 2 μM (Sigma) or 5-aza-2 deoxycytidine (5-Aza2C) 1 - 2 μM (Sigma) were added to the culture for 6 days or 12 days, starting from 1 day of culture or 8 day of culture to examine the effect of DNA methylation on the basal and suprabasal cell layers, respectively.

z-VAD-fmk (Calbiochem), a general caspase inhibitor was dissolved in DMSO to be added to a final concentration of 40 μM.

Mitomycin C (Sigma) was prepared in PBS and added in a final concentration

of 10 µg/ml, 5 µg/ml or 1 µg/ml and added to the culture medium for 24 hrs.

Keratinocyte serum-free medium (KSFM) culture was supplemented with 25 µg/ml of bovine pituitary gland extract (BPE) and 2.5 µg/ml of recombinant epidermal growth factor (EGF) according to the manufacturer's instruction. E11.5 oesophageal explants were cultured in the KSFM medium right after isolation from the embryo for 7 days.

Cyclopamine (Toronto Research Chemicals) was prepared in DMSO and stored in - 20°C as a 250 µM stock, and added in the culture medium to a final concentration of 1 µM from day 1 of culture for 6 days.

20 mM LiCl (Sigma) was added to the culture medium from a 5M stock (dissolved in H_2O). 20 mM NaCl (Sigma) was used as control.

2.3 Histology, immunohistochemistry and immunofluorescence detection

Oesophageal cultures (1-20 days) were fixed for 5 min in acetone / methanol (1:1 ratio) (for immunostaining of cytoskeleton proteins) at room temperature or 30 min in MEMFA (10% formalin, 01% MOPS, pH 7.4, 2mM EGTA, 1mM $MgSO_4$) (for membrane, cytosolic and nuclear proteins) at room temperature. Cultures were then washed three times in PBSA and stored in PBSA at 4°C for several days.

Cultures were permeabilised by adding 1% TritonX-100 in PBSA for 30 min prior to immunostaining. Antigen retrieval was performed if necessary by adding citrate buffer (pH 6) (LAB VISION) for 1 hr at 37°C. The cultures were washed three times for 10 minutes in PBSA. Non-specific binding sites were blocked for at least 1h in 2% Blocking Buffer, which was made up according to the manufacturer's instruction. 5x stock of Roche Blocking Reagent was made

by dissolving with shaking and heating the Blocking Reagent (Roche, 1096176) in maleic acid buffer to a final concentration of 10% (w/v). The stock solution is autoclaved and stored at -20°C before diluting to 2% with PBSA. Primary antibodies were applied overnight at 4°C and the following day the samples were washed three times in PBS for 10 min each. The fluorescent secondary antibody was applied for 3 hrs in the dark at room temperature and the coverslips were rewashed and then mounted in Gelvatol medium. Gelvatol is prepared by dissolving 20 g of polyvinyl alcohol in 80 ml of 10 mM Tris, pH 8.6, and adding 0.2% NaN_3. This is mixed with 50 ml glycerol containing 3 g of n-propyl gallate. Any lumps were removed by centrifugation and the gelvatol was stored in the dark at 4°C.

When more than one primary antibody was used, the second round of staining began after the first round of 2° antibody incubation had been finished. Samples were washed 3 times in PBS before applying the second 1° antibody overnight at 4°C. Samples were washed 3 times the next day and the second 2° antibody then added for 3 hrs at room temperature and the coverslips were washed and mounted in Gelvatol. 0.1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) was applied 15 min to stain cell nucleus when appropriate.

For analysis of *in vivo* changes in expression, tissue was fixed at different stages of development from embryonic E11.5 up to 3 days postnatal and the adult tissue (~2 months old). For staining of transcription factors, tissue was fixed in MEMFA, and for keratin staining, tissue was fixed in acetone/methanol (vol. 1:1). In both cases, the length of fixation varied depending on the size of the sample. For tissue from E11.5 and E13.5 embryos, 2 hrs was sufficient. We used 24 hrs fixation for E15.5, E17.5 and E18.5 embryos and 48 hours for P1, P3 and adult tissues. All fixations were performed at 4°C. After fixation,

samples were washed three times in PBSA. The tissue was then placed in a tissue processor, the typical program was serial immersion in 70%, 90%, 95%, 100% EtOH twice followed by Histoclear (National Diagnostics) twice. Tissues were then embedded in paraffin. All conditions were carried out for 2 hrs. The wax-embedded samples were then positioned in trays and covered with wax, which were allowed to set. Subsequently, transverse sections were cut 7 µm on a Leica 2155 microtome.

For haematoxylin and eosin (H & E) staining of sections, histoclear was used to dissolve the paraffin, and sections were gradually hydrated in 100% EtOH twice, and in 95% EtOH, 90% EtOH, 75% EtOH, 50% EtOH and RO water once, each for 1 min. Haematoxylin was added for 5 minutes followed by 15 seconds of 0.1% HCl treatment to sharpen the contrast then 30 seconds of 0.1% NaOH to neutralise the pH. Eosin treatment was for 30 seconds and slides were then washed, dehydrated in graded alcohol in the reverse sequence of the EtOH used in hydration process and mounted in DePeX (BDH). 3,3-Diaminobenzidine (DAB) staining was carried out by first permeabilising the re-hydrated slides (as done in H&E) with 1% Triton X-100 in PBSA for 30 minutes at room temperature, and then incubated the slides with 0.6% H_2O_2 in 80% methanol, and blocked in the 2% Roche blocking buffer. Slides were then stained with the primary antibody overnight at 4°C. The following morning, the slides were washed with PBSA and the appropriate biotinylated secondary antibody was added for 1 hr. The DAKO ABC detection kit was used for development with the chromogen DAB. One DAB tablet was dissolved in 0.1M Tris, pH 7.5 to a final concentration of 6.67 mg/ml to which H_2O_2 was added to a final concentration of 0.1%. To stop the staining reaction, the slides were washed with PBSA and counterstained with haematoxylin.

Prior to mounting in DePeX, the sections were dehydrated in graded alcohols as in H & E staining and immersed in Histoclear twice.

For dual immuno-histochemical staining, the first round of staining was the same as the single staining procedure, followed by a short microwave (45 seconds on 800 W) and slowly cooling down for 15 min. Sections were then incubated with the second primary antibody overnight. Staining procedure is the same on the next day as single DAB staining using ABC kit from DAKO with an additional round of H_2O_2 quenching and lastly developed in a DAB solution added with $NiSO_4$. (DAB 0.6 mg/ml, 320 mM NiSO4, 0.1% TritonX-100 in a pH 7.2 acetate buffer containing 10 mM imidazole), mounted in DePeX without counterstaining.

The primary antibodies used with sources were as follows, the concentration used was 1/100 unless otherwise stated: mouse anti-GFP, Clontech (8362-1); rabbit anti-GFP, Abcam (ab290); mouse anti-keratin 14, NeoMarkers (MS-115); rabbit anti-K14, Covance (PRB-155P); mouse anti-keratin 18 (LE61) (neat), Prof. E Birgitte Lane, University of Dundee, UK; Rat anti-keratin 8 (TROMA I), Devlopmental Studies Hybridoma Bank (DSHB); mouse anti-pan p63, Santa Cruz (4A4); mouse anti-AP-2α, DSHB (3B5); rabbit anti-C/EBPα, Santa Cruz (sc-61); mouse anti-C/EBPβ, Santa Cruz (sc-7962); mouse anti-Desmin, DAKO (D33); mouse anti-smooth muscle myosin kinase, Sigma (K36); mouse anti-α-smooth muscle actin, Sigma (1A4); mouse anti-skeletal myosin (Fast), Sigma (MY-32); rabbit anti-human Myoglobin, DAKO (A0324); mouse anti-K10, DAKO (DE-K10); mouse anti-Involucrin, Neomarkers (SY5); mouse anti-K4, Sigma (6B10); mouse anti-K13, Sigma (KS-1A3); rabbit anti-loricrin, Covance (PRB145P); rabbit anti-filagrin, Covance (PRB-417P); mouse anti-Ki67, BD Pharmingen (556003); mouse anti-beta-catenin, Transduction Laboratory

(610153); mouse anti-E-cadherin, Transduction Laboratory (C20820).

Various species specific secondary antibodies were used, at 1/100 concentration: Fluorescein Isothiocyanate (FITC) anti-mouse IgG, Vector (FI-2000); FITC anti-rat IgG, Vector (FI-4000); FITC anti-rabbit IgG, Vector (F-0382); Texas Red anti-Rat IgG, Vector (TI-9400); Tetramethylrhodamine Isothiocyanate (TRITC) swine anti-rabbit IgG, DAKO (R0156).

Normally, the secondary antibodies of the same species were applied to specimens as negative controls before trying any primary antibody first time; experiments were usually done for more than twice unless stated otherwise.

Specimens were either examined under a DMRB compound microscope (Leica) and images collected using a SPOT camera with Image Solution software; or collected using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Welwyn Garden City, UK). The same image collecting conditions were applied for cytokeratins expression for the time course results. For determining the area of the mitomycin C treated cultures, the outline of the epithelium region are measured by the peripheral lining function of the confocal imaging program – LSM 5 Image browser. Final images were processed with Photoshop (Adobe System).

2.4 DiI lineage tracing

The E13.5 oesophagi were dissected out, and microinjected with 100 μM of 1 ,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Vybrant DiI solution) (1mM stock from Molecular Probe, V-22885). Final concentration of DiI was prepared by diluting the stock in 90% DMSO and 10% EtOH solvent. Centrifugation may be needed to remove the undissolved crystal. DiI was pipetted into thin wall borosilicate glass capillaries; the parameters for the

capillaries are the same as for electroporation (section 2.9). Oesophagi were then cultured until wanted for live image under confocal microscopy, or to follow up the lineage tracing by immunostaining with markers. Confocal images were taken for the immunostaining; the secondary Ab is conjugated for FITC and combined with DiI, the red fluorescence. The oesophagus cultures were fixed with 30 minutes of MEMFA, and lightly fixed with acetone/methanol for 1 minutes in 4°C before the immunostaining procedure.

2.5 Dead cell assay

Ethidium Homodimer (Molecular Probes) is used a marker for cell death because it will penetrate the cell membrane of dead and dying cells and stain fragmented DNA (Decherchi P. et al., 1997). To stain for dead and dying cells, the medium was removed at different time points and replaced with 2 ml of Hank's Balanced Salt Solution (HBSS without phenol red). Add a final concentration of 1.6 μM Ethidium homodimer to the HBSS. Incubate at 37°C for 20-40 min. Wash with PBS three times, then fix in MEMFA and immunostain with antibodies as designed.

2.6 Fluorescence activated cell sorting

To prepare single cell suspensions of the embryonic organ cultures, I took between 15 and 20 oesophageal buds from each time point. The buds were washed once with PBS, trypsinized for 10 min in the 37°C incubator and then pipetting trituration was performed for 10-15 min. The cells were passed through a 20G needle or a 40 μm cell strainer (Falcon) to ensure single cell suspension. Cells were counted on a haemocytometer and the number adjusted to ~ 10^5 cells/tube with staining buffer (2% FCS, 0.1% NaN_3 in PBS).

The cell suspension was permeablised and fixed by incubation for 15 min with Cytofix / Cytoperm solution (4% PFA, 0.5% Triton X-100, 2% FBS and 0.1% NaN_3 in PBS). The cells were then washed in Perm/Wash buffer (0.5% Triton X-100, 2% FBS and 0.1% NaN_3 in PBS) to maintain the permeability of the cells. Primary antibodies were added at a dilution of 1:200 and were incubated for 30 min in room temperature prior to washing and incubating for 30 minutes with FITC-conjugated secondary antibodies (1:200). Propidium iodide with RNaseA (BD Pharmingen #550825) was added to designated tubes 15 minutes before analyzing on the FACS machine (BD Coulter flow cytometer). Before determining the DNA content of $K14^+$ and $K18^+$ cells, cell samples that had been incubated with secondary antibodies alone and single colour staining were used to establish background signal and the compensation parameters.

2.7 RT-PCR analysis

Total RNA was extracted using the TRI reagent (Sigma). Briefly, embryonic tissues were dissected as described previously at different time points, and collected into 1 ml of TRI reagent and stored at -80°C before further RNA extraction. Embryonic tissue in TRI reagent were homogenised on ice by using a glass dounce homogeniser. After homogenization, the lysate was collected, and then 0.2 ml of chloroform per millilitre of TRI reagent was added to the tube. The tubes were shaken vigorously for 15 seconds, allowed to stand for 10 minutes at room temperature and then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube, and 0.5 ml of isopropanol was added and mixed. The sample was allowed to stand for 5-10 minutes at room temperature, and then centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was

washed with 75% ethanol. The RNA pellet was dried for 5-10 minutes, dissolved in water, and then stored in -80°C.

Before carrying out the cDNA synthesis, the RNA samples were digested with RQ-1 DNase (Promega) to remove any contaminating genomic DNA. First strand complementary DNA was synthesized using Murine Maloney Leukaemia Virus (MMLV) reverse transcriptase (Invitrogen).

The PCR reactions were processed in a DNA thermal cycler under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The primer sequences and the number of cycles are as follow,

MoTF-AP-2α-F544	TATCTACCCCCAGTCGCAAG	351 bp	35x
MoTF-AP-2α-R894	GACACGGGGCCTTTCTTAAT		
m63TA.F#	TCGCAGAGCACCCAGACA	610 bp	35x
m63dN.F#	TTGTACCTGGAAAACAATG	447 bp	35x
m63-2R#	GCATCGTTTCACAACCTCG		
mp63TA*-F	TTTGAAACTTCACGGTGTGC	117 bp	35x
mp63TA*-R	GGCAGATCTGTAATAACTTTCTTTCC		
Mo C/EBPα-F	GTTCCAGATCGCGCACTGCG	449 bp	35x
Mo & Rat C/EBPα-R	TGACCAAGGAGCTCTCAGGC		
Mo & Rat C/EBPβ-F	ACAAGCTGAGCGACGAGTAC	159 bp	35x
Mo & Rat C/EBPβ-R	ACAGCTGCTCCACCTTCTTC		
Mo & Hu K14-F	GTGACCATGCAGAACCTCAA	404 bp	28x
Mo & Hu K14-R	TTCTTCAGGTAGGCCAGCTC		
Mo K18-F	GTGGAATCCAGACCGAGAAA	496 bp	28x
Mo K18-R	GCATCCACTTCCACAGTCAA		

Mo HoxC4-F	GCACTTCTGAAGACCACTCC	331 bp	35x
MoHoxC4-R	CCATGCTAAGACAACACCAC		

(#): primer sequence from (Koster et al., 2004).

2.8 Preparation of plasmids

Cloning of plasmids was carried out by standard molecular biology protocols. To generate the *pK14-nucGFP* plasmid, nucGFP was excised from pcDNA3-nuclearGFP (Tosh et al., 2002) with *BamHI* and*XbaI* and cloned into the same sites of the K14 expression cassette (Vasioukhin et al., 1999) generously provided by Professor Elaine Fuchs, Howard Hughes Medical Institute, Rockerfeller University, New York. The K14 expression cassette contains a 2100 bp of the keratin 14 promoter/enhancer, a rabbit β-Globin 5 untranslated region (UTR), together with an intronic sequence upstream of the *BamHI, XbaI* sites, and the K14 3 UTR, followed by a polyadenylation site 3 downstream of the *BamHI /XbaI* site. Similarly, *pK14-LucE* was generated by excising the K14 promoter/enhancer region from the K14-cassette with *BamHI, SacI* and cloned into the *BglI, SacI* sites of the pGL3-Enhancer vector (Promega) containing the firefly luciferase protein. *pK18mTELucE* was generated by excising the luciferase from the pGL3-Enhancer with *BamHI, NcoI* and cloned into the same sites of the pK18mTELacZ (Chow et al., 2000) (a plasmid kindly provided by J Hu, Lung Biology Research Programme, Research Institute Hospital of Sick Children, Toronto, Canada). The K18mTELacZ plasmid contains a 3.3 kb of the keratin 18 promoter/enhancer region, including the 1st intron of K18 where the splicing site was deleted and a translation enhancer was added (Chow et al., 2000). pGL2-Enhancer is obtained from Promega and used initially as a positive control for Luciferase

assay. pAP-2α, was obtained from MRC geneservice, UK, vector backbone is pCMV-SPORT6. Rat pC/EBPα and mouse pC/EBPβ plasmids were gifts from S. McKnight, University of Texas Southwestern Medical Center, and subcloned in the pcDNA3 multiple cloning site by using HindIII and BamHI restriction enzymes. Human p63 isoforms pTAp63α, pΔNp63α and pTA*p63αwerein the pcDNA3.1/mycHis plasmid backbone, they are gifts generously provided from K. Engeland, Max-Burger-Forschungszentrum, University of Leipzig. pcDNA3 was obtained from Invitrogen.

2.9 Electroporation of plasmids into embryonic organ cultures

Briefly, 0.1% Fast Green was added to 1 μg/μl of plasmid DNA for easy visualisation and this was pipetted into thin wall borosilicate glass capillaries. The parameters for pulling the capillaries on a Micropipette Puller (Sutter, P-97) were: Heat 300, Pull 40, Velocity 220, Time 150. The capillaries had the following dimensions: outer diameter, 1.0 mm, inner diameter, 0.58 mm (Clark Electromedical Instrument, GC100-15). The electrode gap width was 5mm. For electroporation, I used the Electrosquareporator (BTX, ECM830), with the voltage set at 50V, the pulse length 50 ms, continuous electroporation for 3 times in a unipolar direction, interval was 200ms. DNA was injected into the lumen of the oesophagus, stomach, trachea-lung buds and small intestine that had been dissected free in PBSA. Tissues were then immunoassayed after 24h (oesophagus) or 48h (stomach, trachea-lung buds and intestine) of embryonic explant culture.

2.10 Assay of luciferase activity

The purpose of luciferase assay is to determine whether transcription factors

will activate or suppress the expression of a reporter gene linked under the control of a piece of genomic DNA containing the promoter/enhancer region of interest. Briefly, KYSE30, OE33, CaCO2 and NIH3T3 4.2 cell lines were seeded at ~ 40 - 50% confluency into 6-well plates 24 hours before transfection. Cells were then transiently transfected by using 6 µl GeneJuice with 1 µg of a promoter drive firefly luciferase reporter plasmid (pK18mTELucE, pK14-LucE, pTTR-Luc), 2 µg of transcription factor(s) including pcDNA3 with pAP-2α, pC/EBPα, pC/EBPβ, pTAp63α, pTA*p63α, pΔNp63α or different combinations of two p63 isoform plasmids, and 1 µg of β-Gal plasmid (pCMV-nucLacZ) to normalise for variations in transfection efficiency. Initially, a constitutive luciferase expressing plasmid, pGL2-Enhancer (Promega) was used as a positive control for the assay system (data not shown). After 48 hours of incubation, cells were harvested for measurement of luciferase activities by using the Luciferase Assay Reagent (Promega). Light emissions were integrated for the initial 10 seconds of emission detected by Microplate Luminometer (Berthold LB96V) and operated by Winglow software. β-Gal activity and protein quantity were assayed by a Microplate Colorimeter (Anthos AN2001) and Stingray v1.5 software. I used 2-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma N1127) as the substrate in the β-Gal assay; Biorad Protein Reagent (500-0006) and BSA standards were used for protein assay. For the β-gal assay, 140 µl of 0.1 M PO4 buffer with 10 mM of KCl, 1mM of $MgSO_4$ and 50 mM 2-mercaptoethanol were added to a 20 µl of sample and 25 µl of 4 mg/ml ONPG in the 96 well plates, incubated at 37 °C overnight before β-Gal measurements were done on the Microplate Colorimeter using the absorbance 405 nm filter. For the protein quantification, 190 µl of five times diluted Biorad protein reagent (in H_2O) were

added to 10 µl of sample, waited for 30 minutes in room temperature and the protein measurement was done by the Microplate Colorimeter reading at the absorbance of 590 nm. β-gal assay and protein assay were done in duplicates. The luciferase specific activity was determined by subtracting background signal and normalizing to the β-Gal activity and protein quantity of each sample. A standard curve of Luciferase standard ranging from 1 ng ~ 10^{-4} ng was used to determine the definitive luciferase quantity and to compare values between each different experiments. Fold induction (or inhibition) was calculated by dividing the values obtained for the transcription factor(s) by the values of control plasmid (pcDNA3). Samples were run in quadruplicate, and all experiments were performed on at least three separate occasions.

2.11 Statistical Analysis

Statistical differences were analyzed with a 2-way Student-t test. All tests were assessed at the 0.05 level of significance.

Chapter 3 *In vivo* and *in vitro* development of the oesophagus

3.1 Introduction

3.1.1 Overview

To date, study on the development of the oesophagus is scarce. The reason for this is partly due to a lack of suitable model system. Except for some oesophageal cell lines (e.g. OE33, KYSE30) that do not fully reflect the characteristics of the organ, there is no proper *in vitro* model for the study of the oesophagus. Here, the development of the oesophagus *in vivo* and the establishment of an *in vitro* culture system will be described. Some *in vitro* culture systems commonly used for mammalian organ explant are briefly introduced.

3.1.2 In vitro culture systems

3.1.2.1 The need for an in vitro culture system of the oesophagus

The aim of this chapter is to describe the establishment of an *in vitro* culture system for the oesophagus. A culture model system may facilitate the understanding of oesophagus development which might not be achieved from *in vivo* models. The development of such a model will help us gain more understanding of oesophageal development. Information such as (a) the mechanism underlying formation of the endodermal stratified squamous epithelium, (b) the maturation of the mesenchymal and musclar tissue, (c) the interactions between the mesodermal and endodermal tissues could be achieved by a suitable culture system. An additional benefit of an *in vitro* system is that it can also be manipulated, e.g. addition of extracellular factors

and ectopic expression of genes. The frequently used methods for *in vitro* culture are described below.

3.1.2.2 Features of different in vitro culture systems

Commonly used culture systems include (a) culture of a single cell suspension, (b) hanging drop culture of cells or organs and (c) whole-mount organ culture on extracellular matrix coated cover-slip. It is known that cell-cell contact, cell adhesion to the extracellular matrix in the basal lamina, and aggregation, sorting and migration of different cell types in the embryo are important factors involved in the social context of individual cells during morphogenesis and organogenesis. These factors regulate the survival, proliferative and differentiating signals of cells, and are involved in how organs take shapes and function. Different culture methods will influence the aspects of these important factors.

The culture of cells following mechanical or enzymatical digestion is a method advantageous for gene delivery (e.g. easy for transfection) and is straight forward for immunostaining steps of the samples. However, the disadvantage is that the normal relationship of the different cell types in a tissue is usually lost. However, many detail information of molecules and mechanisms that regulate cellular differentiation was found using single cell culture methods. Such models are also useful in verifying gene function. For instance, clonal-derived lens epithelial cells isolated from newly hatched chicks show differentiation when cultured in conditioned medium (Okada et al., 1973). There is also the study of cadherin gene function in cell-cell adhesion and cell sorting using L cells, a line of cultured mouse fibroblasts which do not normally express the classical cadherins. When L cells were transfected with

E-cadherin, they start to adhere to one another in a Ca^{2+}-dependent manner (Nagafuchi et al., 1987). When L cells transfected with N-cadherin were mixed with cells transfected with E-cadherin, the N-cadherin cells would be sorted out from the E-cadherin positive cells. The single cell culture method is the simplest way to understand how individual cell types would behave autologously. Various cell lines now used in labroratories were found using this approach.

Another frequently used method is to culture cells or a piece of tissue in a drop of medium suspended on an inverted solid support (the "hanging drop" method) (Gilbert, 2000). The surface tension of the drop of medium keeps the small tissue from free falling. Although there are usually no dramatic changes in the size of the culture, the tissue (or cell spheroid) formed will usually differentiate. The hanging drop method was originally developed by Ross Harrison to study the identity and formation of neuronal axon (Gilbert, 2000). He cultured the neural tube of a frog tadpole in a drop of frog lymph on a transparent inverted coverslip and observed the development of axon outgrowth at a speed of 56 μm/hr from the neuroblast in the cultured neural tube. The hanging drop method is now widely used for the study of culturing embryonic stem cells to a spheroid differentiated structure, the embryoid body (Yamada et al., 2002). The advantage of using hanging drop culture is in the maintenance of the 3D structure of the tissue or embryonic organ. Physiological intercellular interactions and cellular organization can be readily studied by hanging drop model, such as understanding the vascularizion of a microtissue (Kelm et al., 2004). However, long-distance migration of cells during development is not easily studied by this method.

The whole-mount organ culture on different extracellular matrices on a solid

coverslips has been adopted in our lab to investigate the development of pancreas (Percival and Slack, 1999). The characterisitc of this method is that morphologically, the tissue or organ in culture is intact, but gradually it becomes stretched and flattened while lying on the surface of the coverslip. The spatial organisation of cells in the culture is mainly the same as *in vivo* although the direction of the mechanical stress applied on the culture might be different. The cell layers in direct contact to the extracellular matrix pre-coated on the coverslip also encounters an articial environment initially but later these cells will secrete their own extracellular proteins. However, the epithelial cells in the pancreas culture model and the oesophagus model shown here do express the differentiated phentotype.

In the current study, the method generally adopted to investigate the development of the oesophagus is the whole-mount culture on the extracellular matrix coated coverslips. Next, previous work on the oesophageal development is reviewed.

3.1.3 Development of the oesophageal epithelium

In humans, pseudostratified columnar epithelial cells initially line the embryonic oesophagus (DeNardi and Riddell, 1991). At 8 weeks of gestation, ciliated cells can be found in the middle third of the oesophagus and extending rostrally and caudally. At 10 weeks, a single layer of columnar cells populates both the proximal (near the stomach) and distal ends of the oesophagus. Around 4 months of gestation, the columnar cells grow downward into the lamina propria, proliferate and then differentiate into the cardiac-type glands of the oesophagus. At approximately 5 months gestation, stratified squamous epithelium initially appears in the middle third of the oesophagus and extends

towards the rostral and caudal ends, replacing the ciliated epithelium (Johns, 1952). The process of *in vivo* epithelial differentiation has not been studied further, but development of human foetal oesophageal explants from ~12-16 weeks gestation have been investigated *in vitro*, and an accelerated rate of formation of stratified squamous epithelium were found after 15 days in organ culture (Menard and Arsenault, 1987).

3.1.4 Cytoskeletal proteins: intermediate filaments and cytokeratins

Intermediate filaments are cytoskeletal polymers that provide structural support to the cytoplasm and nucleus of higher eukaryotes; they also function to sustain the cells for mechanical and non-mechanical stresses (Coulombe and Wong, 2004). Intermediate filaments such as keratin or desmin connects to desmosomes in cell-cell anchoring junctions via cytoplasmic plaques composed of intracellular dense anchor proteins - desmoplakin and plakoglobin (γ-catenin) (Jamora and Fuchs, 2002). A desmosome is the anchoring cell-cell junction found between two epithelial cells, and consists of the transmembrane proteins desmoglein and desmocolin of the cadherin family. In cells anchoring to the extracellular matrix, intermediate filaments connect to the hemidesmosome via anchor protein – plectin, which binds to integrin in the cell membrane hemidesmosome. Integrin binds to basement membrane extracellular matrix such as laminin and facilitates the anchoring of cells in the cell-matrix adhesion.

Actin is also a major cytoskeletal protein which facilitates the intracellular cytoskeletal support to the cell-cell adherin junctions and the cell-matrix focal adhesions. The adherin junction proteins-cadherins, such as E-cadherin expressed in the epithelial cells, facilitates the calcium-dependent binding of

two neighbouring cells. Actin filaments are connected to the membrane cadherins by associating with anchor proteins vinculin, α-actinin and α, β and γ-catenin or p120. In the focal adhesion complex, actin connects to integrins via anchor proteins such as talin, vinculin, α-actinin and filamin.

Structural components such as keratin filaments, anchoring prepteins and desmosomes form a three-dimensional array within the cytoplasm of epithelial cells. Recent findings showed that keratins interact with non-structural proteins that influence cell growth and cell death (Coulombe and Wong, 2004). The intermediate filaments belong to a large family of structural proteins and are expressed according to the differentiated state of the cell (Lazarides, 1980). For example, keratin 18 (K18, a type I acidic cytokeratin intermediate filament protein), normally heterodimerizes with another simple columnar intermediate filament (the type II cytokeratin 8 (K8)) in simple columnar cells (Owens and Lane, 2003).

3.1.5 What are the markers of an oesophageal stratified squamous epithelium?

In stratified squamous tissue, structural proteins maintain the integrity of epithelial tissues and function as a protective barrier. As part of the normal growth of stratified squamous tissues, terminally differentiated cells are continuously sloughed off the cornified layer and are replaced from cells of the basal layer. Cytokeratin 14 (K14) and cytokeratin 5 (K5) are markers for the basal layer of stratified squamous tissue. Mutations in keratins K5 or K14 are found in epidermolysis bullosa simplex (EBS), the abnormal keratin filament aggregates in the basal cells and disrupts the keratin filament cytoskeleton complex, resulting in cells that are less resilient and cause blistering when

subjected to even mild physical trauma (Smith, 2003). K1 and K10 are specific markers for the suprabasal layers. Bullous congenital ichthyosiform erythroderma is caused by mutations in keratins 1 and/or 10. These patients are born as bright red babies with large blisters and erosions. Other tissue specific cytokeratin markers are such as K4 and K13 for the oesophagus, cornea and buccal epithelium and function-specific cytokeratins such as K6 and K16 for wound healing of epithelia cells (Moll et al., 1982). The cornified cell envelope is an extremely tough protein / lipid polymer structure formed just below the cytoplasmic membrane and subsequently resides on the exterior of the dead cornified cells. Loricrin, a terminal differentiation marker of the stratified squamous, is the major protein of the cornified cell envelope, a structure that replaces the plasma membrane during keratinocyte terminal differentiation (Nemes and Steinert, 1999; Presland and Dale, 2000). Loricrin and involucrin are proteins cross-linked into the cornified layers by the transglutaminase enzyme. Filaggrin is also a terminal differentiation marker, and is expressed in the corneum stratum of stratified squamous tissue such as epidermis and oral cavity. A defect in pro-filaggrin causes ichthyosis vulgaris, which shows fine white flaky scales of the extensor surfaces, trunk, flank, lower legs but spares the folds and wet areas (Presland et al., 2000). Autoimmune antibodies reactive to filaggrin is a marker for rheumatoid arthritis and could be involved in the pathogenesis of this disease (Francis, 1994; Serre, 2001). Using a combination of the cytokeratin markers described above should provide detailed information on the development of the oesophageal epithelium. Table 3-1 and Figure 3.1 shows the keratin markers I used in the present study.

Table 3-1 Cytokeratin markers and expression profile used in this study:

Markers	Expression profile
K8, 18	Columnar (glandular) epithelium
K5, K14	Basal layer of stratified squamous epithelium
K4, K13	Oesophagus, buccal and cornea specific epithelium
K1, K10	Early differentiated marker of the suprabasal stratified squamous epithelium.
Involucrin	Earliest differentiation marker located in the suprabasal stratified squamous epithelium.
Loricrin	Late / terminal differentiation marker located in the spinal to cornified layers of the oesophageal stratified squamous epithelium.
Filaggrin	Late / terminal differentiation marker located in the granular to corneum layers of the oesophageal stratified squamous epithelium.

*:after Moll, R., et al. (1982). *Cell* 31, pp11-24.

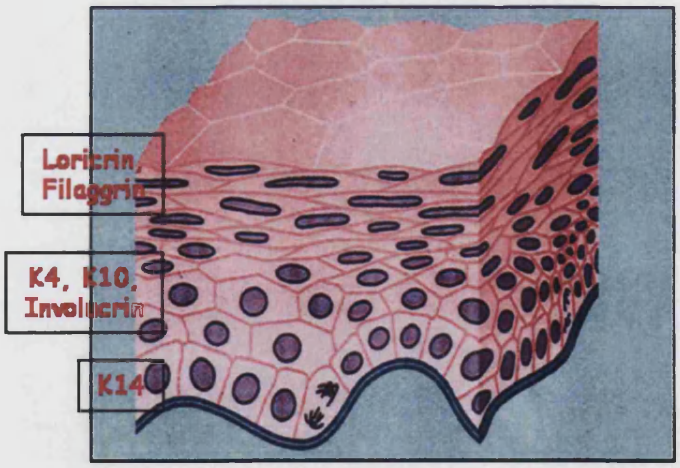


Figure 3.1 A diagram showing the relative location of distinct types of cytokeratin-expressing cells in each layer of stratified squamous epithelium (adapted from Young and Heath, 2001).

3.1.6 Development of the oesophageal muscle

As stated previously (Chapter 1, section 1.2.3), the adult mouse oesophagus contains both smooth muscle and skeletal muscle cell types (Patapoutian et al., 1995; Kablar et al., 2000). Skeletal muscle is found predominantly in the upper two-thirds and smooth muscle in the lower third of the mammalian oesophagus. It was shown that prior to E15, the myogenic regulatory factors (MRFs) of skeletal muscle are not expressed in the mouse oesophagus (Kablar et al., 2000). This raises the question of where the skeletal muscle cells come from? The origin of the skeletal muscle was thought to come from smooth muscle cells and that the conversion was classified as transdifferentiation. However, there are some contradictory findings. By immunohistochemistry and electron microscopy, Zhao and Dhoot suggested that there are individual smooth and skeletal precursors located in the foetal oesophagus that follow different differentiation pathways and the conversion is not transdifferentiation (Zhao and Dhoot, 2000). There is also a report using lineage tracing in mouse expressing two proteins driven by the smooth muscle specific promoter (smooth muscle heavy chain myosin (smMHC)) (Rishniw et al., 2003). One encodes enhanced green fluorescence protein (eGFP) for real time monitoring of smooth muscle expression, and the other is the Cre recombinase expression for tracing cells that have ever activated the smooth muscle gene. This line is mated with the reporter line R26R, which is a mouse line containing a CMV promoter driving a β-galactosidase gene with a loxP-stop-loxP site at its 5 region. The CMV promoter will only be able to drive LacZ expression when Cre recombinase reaches a certain level in the same cell. By this approach, the authors are able to differentiate between smooth muscle cells

that are currently expressing smMHC, (GFP and LacZ positive) with those cells that have expressed the smMHC gene previously, but not any more (only LacZ positive) due to transdifferentiation. From the crossed smMHC-Cre:GFP x CMV-loxP-stop-loxP-LacZ mice, the authors found that the expression pattern of LacZ is exactly the same as the GFP in the oesophagus, indicating that the skeletal muscle in the oesophagus must not come from the smooth muscle but probably is an outgrowth from a different precursor cell (Rishniw et al., 2003). In this chapter, I will characterise the development of the oesophageal muscle both *in vivo* and *in vitro*, and address whether there is transdifferentiation occurring in the muscle cells.

3.2 The *in vitro* culture system of the oesophagus

3.2.1 Development of an in vitro culture system for oesophagus

To address some questions of the development of the epithelial and muscle layers encircling the upper gastroenteral tract, oesophagi were dissected free from E11.5d mouse embryos and placed in culture on fibronectin-coated glass coverslips. The size of the dissected oesophagi ranged from 75 µm – 200 µm. After 24hr of culture, the oesophagus had attached to the substrate and the cut ends had healed. At this stage, the culture is composed of a tube of epithelium surrounded by mesenchyme composed of fibroblasts and probably some endothelial cells. During the first few days of culture, some of the mesenchymal cells spread away from the explants but they also continue to maintain a layer above and below the epithelium, which will often spread freely onto the surface of the coated extracellular surface or even straight onto the glass after some days of culture. Results are shown from confocal z-stack images of E-cadherin and smooth muscle actin stained culture summarised in a diagram, Fig 2.1 C. Later in the culture period, the mesenchymal cells resolve into an inner region composed of smooth muscle actin-positive cells and a peripheral region of fibroblast-like cells, resembling the situation *in vivo* (data not shown). The cultures remain viable and continue to grow for at least 20 days although the growth is not as extensive as that found *in vivo*.

3.2.2 Optimizing the culture system using different extracellular matrices

The initial goal was to determine whether spatially and temporally, oesophageal explants differentiate as *in vivo* oesophagi. To find out the conditions for culturing explants, I tried culturing either on glass coverslips or on coverslips soaked with 40 µl of different extracellular matrices solution

dissolved in sterile water (fibronectin, collagen I, collagen IV and laminin), each at a concentration of 50 μg/ml. Most explants attach and epithelium and muscular differentiation occured on glass coverslips as well as on the different extracellular matrices I used (results tabulated in Table 3-2). Although there were some differences as to when the cytokeratin markers become expressed, they were quite small. I found explants usually attached more efficiently to fibronectin, and in this culture system, the muscular and epithelium differentiation rates were also comparable to *in vivo* results. In the present study, I mainly focused on the culture system using fibronectin as a substrate for the oesophageal explants.

Below are expression results of differentiation markers when cultured on different extracellular matrices summarised in a table.

Table 3-2 Epithelial markers expression profile of oesophagi cultured on glass or on different extracellular matrix coated coverslips

	Glass *(4/6)	Fibronectin (6/6)	Collagen I (4/5)	Collagen IV (2/5, many cultures detached)	Laminin (4/5, flower shape of culture)
K8/K18	day1 day14	day1 day15	day1 day14	day1 day15	day1 day15
K14	from day1	from day3	from day 3	from day3	from day 3

*: number of attached and positively staining results out of the number of cases used.

3.3 Conversion of columnar to stratified squamous epithelium in the oesophagus

3.3.1 Morphology of the oesophageal epithelium

Histological sections of mouse oesophagus at different stages of development (from embryonic E11.5 - E17.5 days, perinatal stages and adult) were stained for haematoxylin and eosin (Fig 3.2). Just after the foregut separates into the oesophagus and trachea, the oesophagus is lined with simple columnar epithelial cells surrounded by undifferentiated mesenchyme (E11.5d, Fig 3.2 A). As the oesophagus develops (E13.5-E17.5, Fig 3.2 B, C, D), the epithelium remains columnar but gradually becomes composed of more layers, while the mesenchyme separates into zones corresponding to the later submucosa and smooth muscle layers. At E17.5, the cell layer closer to the lumen starts to show the squamous cell morphology (Fig 3.2 E). In the perinatal stages, a cornified layer of squamous tissue appears (Fig 3.2 F, G). Finally, in the adult, the basal layer is clearly distinct (dense layer of cells) and the suprabasal part is divided into the spinous, granulous and cornified layers of a fully differentiated and keratinised stratified squamous epithelium (Fig 3.2 H, I).

3.3.2 Immunohistochemical analysis of the oesophagus epithelium in vivo

To find out if the expression of the cytokeratins correlates with the morphology of the oesophagus, immunohistochemical analysis was performed to determine the temporal expression of the columnar marker (K8) and stratified squamous epithelium marker (K14) during oesophageal development. At E11.5, all the cells in the epithelium of the oesophagus are K8 positive and K14 negative (Fig 3.3 A, I). K8 continues to be expressed as the epithelium becomes multilayered, until it decreases in the basal layer from E15.5 and

A
E11.5
bm
B
E13.5
bm
m
C
E15.5
m
lu
out
D
E17.5
m
E
m
F
P1
m
G
P8
H
Adult
sm
m
I
Gr
Sp
B
O

Fig. 3.2 Haematoxylin and eosin staining of the mouse embryonic and adult oesophagus.

Haematoxylin and eosin (H&E) staining of the mouse oesophagus during different stages of embryonic development and in the adult. (A) E11.5, (B) E13.5, (C) E15.5, (D, E) E17.5 (F, G) P1 and (H, I) adult (2 months old). The epithelium of the oesophagus at E11.5d is simple columnar and gradually becomes stratified during development. The four distinct layers, i.e. B, basal layer, Sp, spinous layers, Gr, granular layers and C, corneum stratum, features of a fully differentiated stratified squamous tissue are shown in the adult (I). The muscle layers of the oesophagus are found from E13.5, two layers of the muscle (inner longitudinal and outer circular) could be found in the oesophageal cross sections after E15.5, but striations could only be found from after E17.5. bm, basement membrane (a black line drawn in A,B), ep, epithelium, sm, submucosa m, muscle layers, In, inner longitudinal, and Out, outer circular muscle. Scale bars are 60 μm in (A, B, C, E, G, I), 190 μm in (D, F) and 380 μm in (H).

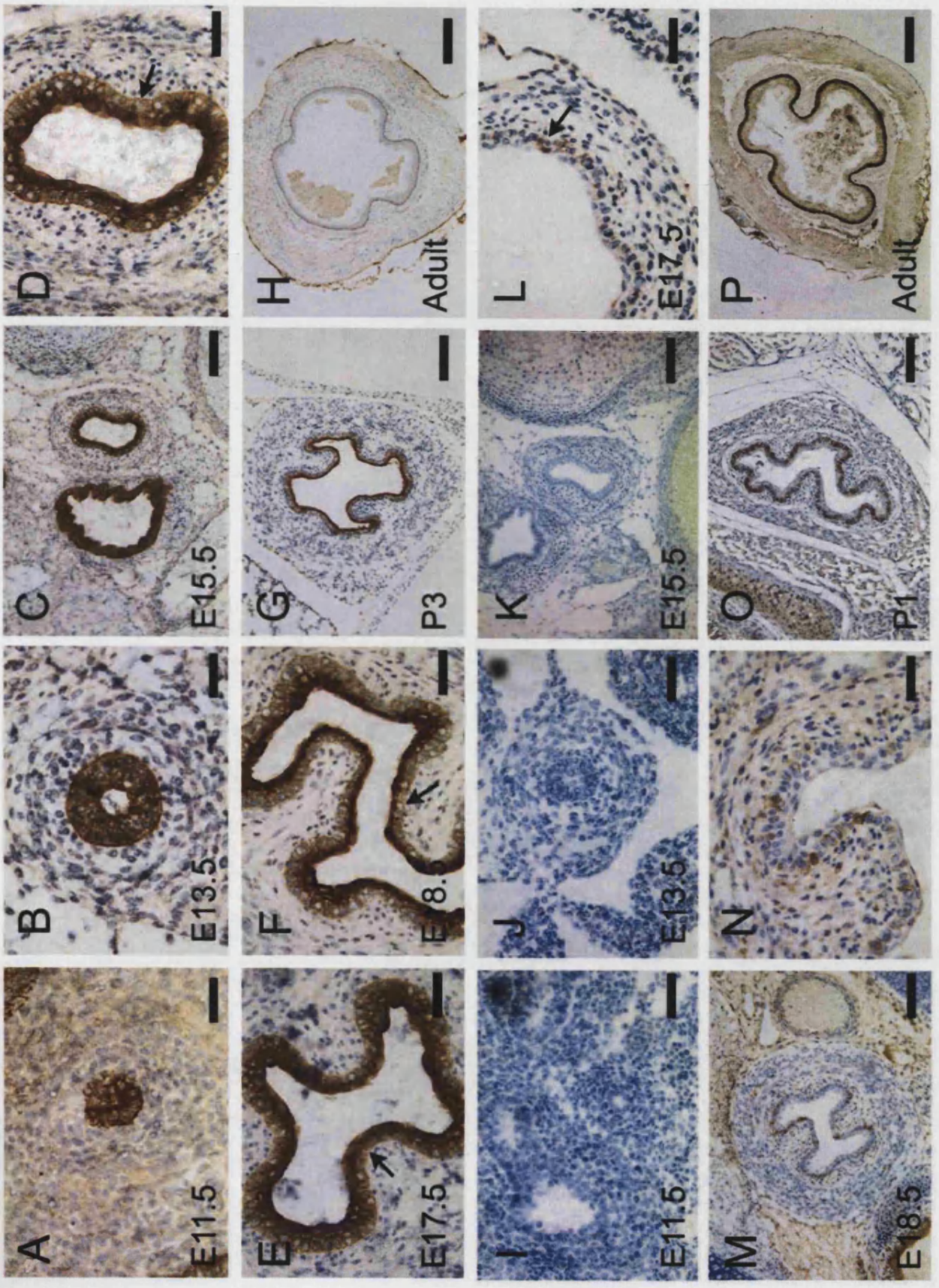

A
E11.5
B
E13.5
C
E15.5
D
E
E17.5
F
E18.5
G
P3
H
Adult
I
E11.5
J
E13.5
K
E15.5
L
E17.5
M
E18.5
N
O
P1
P
Adult

Fig. 3.3 The oesophageal epithelium changes from columnar to stratified squamous in vivo.

Keratin 8 (A-H) and Keratin 14 (I-P) immunostaining on embryonic oesophageal cross sections. (A, I) E11.5, (B, J) E13.5, (C, D, K) E15.5, (E, L) E17.5, (F, M, N) E18.5, (O) postnatal day 1, (G) postnatal day 3, (H, P) Adult (3 months). Keratin markers show a reciprocal expression in the columnar and the basal layer of stratified squamous during the development of the oesophagus epithelium. Arrows in (D-F) show the gradual disappearance of K8 expression in the basal layer, while the arrow in (L) shows the emerging K14 positive cells. Note that the suprabasal epithelium still expresses K8 until P3 but not in the adult. Scale bars are 50 μm in (A, B, D, E, F, I, J, N), 160 μm in (C, G, K, L, M, O) and 320 μm in (H, P).

E17.5 (arrows in Fig 3.3 D, E). It is almost absent from the basal layer by E18.5 (arrow in Fig 3.3 F). However, the suprabasal layer tissue continues to express K8 (Fig 3.3 F, G) until at least P3, only finally disappearing later (Fig 3.3 H, an example of a 2 month old mouse oesophagus). The expression pattern of the K14 stratified squamous marker is quite different. Basal cells expressing K14 could be observed at around E17.5 (arrow in Fig 3.3 L). At P1, most cells of the basal layer express K14 (Fig 3.3 O) and in adult oesophagus, all cells of the basal layer clearly show K14 expression (Fig 3.3 P). The expression of K8 and K14 in the basal layer therefore correlates with the switch from columnar to squamous morphology. Other stratified squamous-specific keratin markers showed similar timing of expression in the appearance of a stratified squamous epithelium (Fig 3.5, Fig 3.6 and Table 3-3). Since K14 is not seen in the suprabasal layers, it presumably turns over fast enough to become degraded before cells move from basal to suprabasal levels. Conversely the fact that K8 does persist in the suprabasal levels of the foetal stages (Fig 3.3 D-G) suggests that it is still expressed in these cells after being turned off in the basal layer.

3.3.3 Immunofluorescence analysis of the oesophagus epithelium in vitro

The *in vitro* culture system was used to compare the time course of the process from simple columnar to stratified squamous cell type to the normal oesophageal development. 24-72 hours after the start of the culture, the entire epithelium was K8 positive (Fig 3.4 A, B). After 5 days of culture the basal layer showed a marked decrease in the number of K8 positive cells (Fig 3.4 C, D) while the K8 positive cells in the suprabasal layers disappeared more gradually, only finally disappearing by about 20 days of culture (Fig 3.4 E-H). To confirm

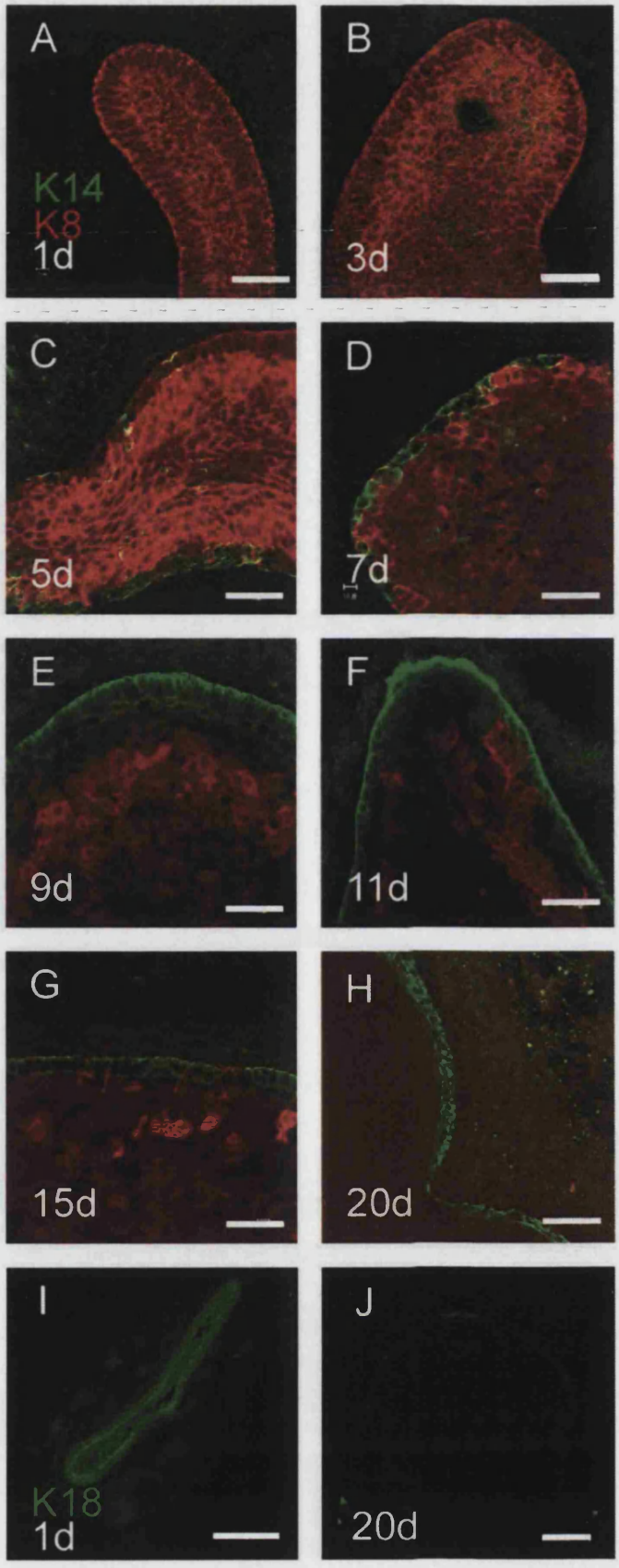

A
K14
K8
1d
B
3d
C
5d
D
7d
E
9d
F
11d
G
15d
H
20d
I
K18
1d
J
20d

Fig. 3.4 *In vitro* evidence for the E11.5 oesophageal culture converting from columnar to stratified squamous.

Oesophageal segments were isolated from E11.5 day embryos, cultured for different times and then immunostained for K14 (green) and K8 (red) or K18 (I, J). (A, I) 1 day, (B) 3 days, (C) 5 days, (D) 7 days, (E) 9 days, (F) 11 days, (G) 15 days and (H, J) 20 days of culture. Scale bars indicate the magnification. The columnar marker K8 was expressed in the epithelium from the first day of culture, whereas the stratified squamous marker K14 only started to be expressed at the basal layer of the epithelium from around 3 to 5 days in culture. The K8 expression becomes less intense in the basal layers at about the same time as K14 cells appear. A second wave of the loss of K8 cells was observed in the suprabasal layers from around 11 days onwards. By 20 days of culture very few K8 positive cells remain. K18 immunostaining on 1d (I) and 20d (J) show the same results as K8 staining. Scale bars are 50 μm in (A-H) and 200 μm in (I, J).

that this result reflected a switch of cell type rather than just of two specific gene products, I also examined K18 expression. This showed a similar pattern and timing of expression as K8 with initial uniform expression but almost complete loss by 20 days (Fig 3.4 I, J). Conversely, the expression of the squamous marker K14 starts in the basal layer by day 5 of culture (Fig 3.4 C) and by 7 days, nearly all cells of the basal layer express K14, and continue to do so during the culture period (Fig 3.4 D-H). Comparing the suprabasal cells stained with K8 on day 1 (Fig 3.4 A), day 7 (Fig 3.4 D) and day 9 cultures (Fig 3.4 E), the suprabasal cells can be clearly seen to gradually change shape from a columnar to a flattened (squamous) morphology in the *in vitro* culture system. This indicates there is a correlation of the appearance of K14 marker and the stratified squamous morphology. Culturing the embryonic oesophagus on different extracellular matrix proteins (including glass) showed the same columnar to stratified squamous conversion (K8 to K14) (data not shown). The study of K8, K18 and K14 indicates that the epithelium of the *in vitro* cultures showed autonomously the same reciprocal expression of cytokeratins as it happens during development *in vivo*.

3.3.4 Other stratified squamous markers

To follow the progress of oesophageal epithelial differentiation, I examined the expression of different markers including involucrin, K10 (Fig 3.5), loricrin, filaggrin and K4 (Fig 3.6). The intermediate filament involucrin (Inv) is an early differentiation marker and cytokeratin 10 (K10) a later differentiation marker for stratified squamous tissue. The expression of K10 (Fig 3.5 A, B) and involucrin (Fig 3.5 G, H) in relation to K14 in the adult oesophagus is shown. K10 or involucrin positive cells are red and are located at the suprabasal layers and

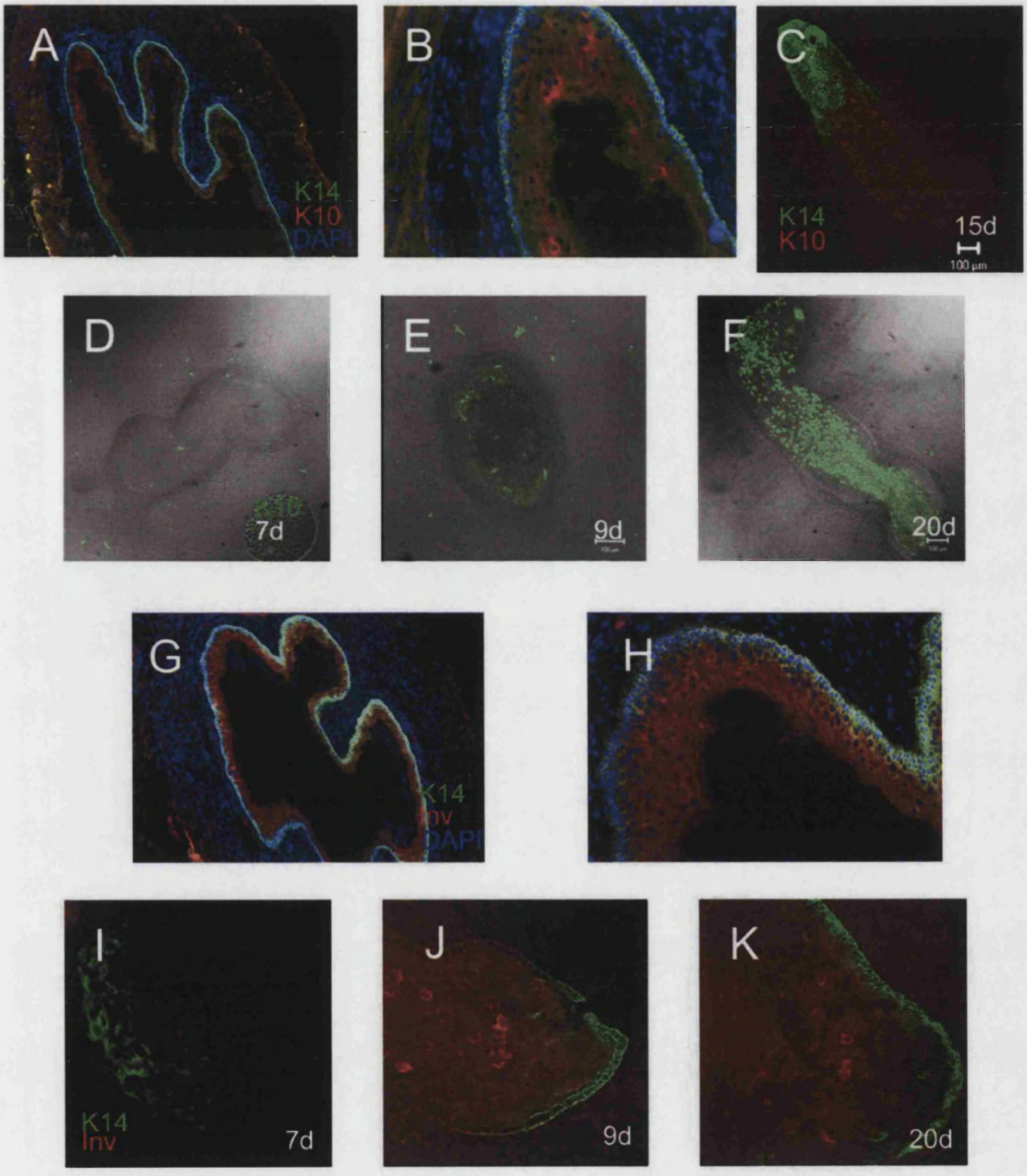

A
K14
K10
DAPI
B
C
K14
K10
15d
100 μm
D
7d
E
9d
F
20d
G
K14
Inv
DAPI
H
I
K14
Inv
7d
J
9d
K
20d

Fig. 3.5 Expression of stratified squamous differentiation markers in adult oesophagus and oesophageal cultures.

K10, a suprabasal marker for the differentiated stratified squamous epithelium was co-stained with the basal layer marker K14. Low (x100) (A) and high (x400) (B) magnification adult oesophageal sections or a 15 day oesophageal culture (C) (K14, green, K10, red in A, B, C) are shown. Invoulcrin, also a suprabasal marker for the terminal differentiation stratified squamous, was co-stained with basal layer marker K14 on adult oesophageal sections and viewed at low (x100) (G) and high (x400) (H) magnification. E11.5 oesophagi cultured for 7 days (D, I), 9 days (E, J) and 20 days (F, K) were stained with K10 (green) (D-F) or co-stained with involucrin (red) and K14 (green) (I-K). 4', 6 -Diamidino-2-phenylindole (DAPI) was used to visualise the cell nucleus (A, B, G, H).

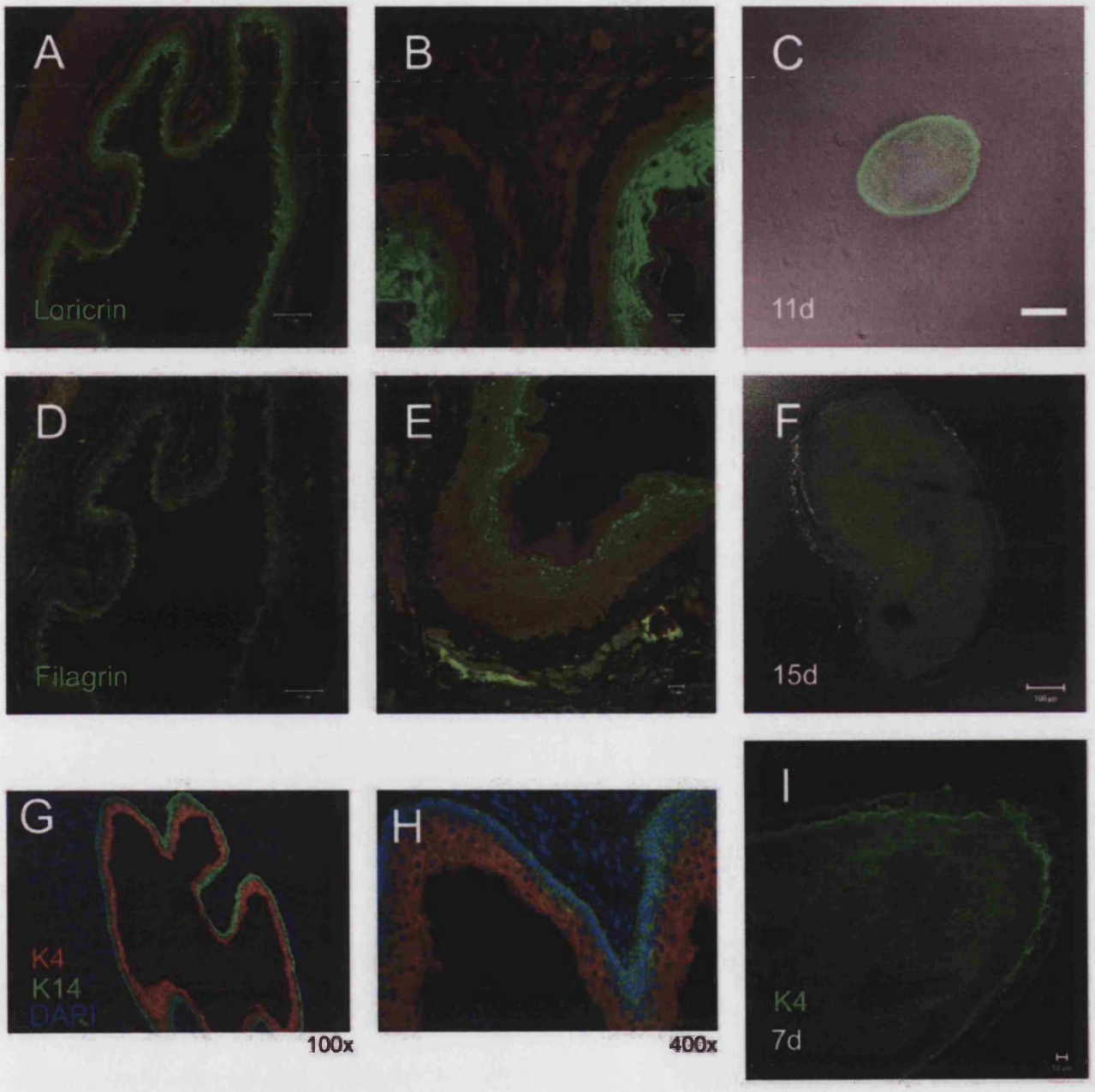

A
Loricrin
B
C
11d
D
Filagrin
E
F
15d
G
K4
K14
DAPI
100x
H
400x
I
K4
7d

Fig. 3.6 Late differentiation and oesophageal specific stratified squamous epithelium markers.

Cross-sections of adult mouse oesophagus (A, B, D, E, G, H) or cultured oesophagus from E11.5 (C, F, I) were immunostained for (A-C) loricrin (green); (D-F) filagrin (green); (G, H) K14 (green), K4 (red) and DAPI; and (I) K4 (green). The culture periods of the oesophageal cultures were (C) 11 days, (E) 15 days, (G) 7 days. K4 expression *in vitro* showed the cultures are oesophageal in character. The expression of loricrin and filaggrin showed terminal differentiation markers of a stratified squamous tissue and usually stained the corneum layers. The magnification of G is 100x and H 400x. Scale bars are 100 μm in A, C, D, F, and 10 μm in B, E, I.

the K14 positive green cells are at the basal layer. The cultured oesophageal explants co-ordinately express K10 (Fig 3.5 C) and involucrin (Fig 3.5 J, K) in the suprabasal layers, which is identical to the normal staining pattern on adult sections. K10 starts to be expressed around 7-9 days of culture but not earlier (Fig 3.5 D-F). Involucrin starts to express at similar time points with very few involucrin cells to be found before 7 days of culture (Fig 3.5 I-K). The K14 positive cells express earlier than involucrin.

To examine whether the oesophageal culture could undergo terminal differentiation, filaggrin and loricrin, two stratified squamous markers located in the suprabasal region most distant to the basal layer (the *corneum stratum*) was used (Fig 3.6 A, B, D, E). These markers were found in the oesophageal explants after many days of culture (Fig 3.6 C 11 days, Fig 3.6 F 15 days), the time points of expression is later than other stratified squamous markers. Lastly, to prove that oesophagus (as opposed to other tissues e.g. stomach or lung) was cultured *in vitro*, K4, (a suprabasal marker for oesophagus and corneum epithelium), was immunostained (Fig 3.6 G-I). The expression of stratified squamous markers with the combination of K4 ensures what was cultured is oesophagus but not trachea (which is pseudostratified and will not express stratified squamous markers such as K14 and K10). These results tell us that the explants isolated at 11.5 days do have the potential to develop into mature oesophageal stratified squamous tissue.

3.4 The oesophagus as a model for developing and transdifferentiating muscular tissue

3.4.1 Morphology of embryonic and adult oesophageal muscular structure

The results of H & E staining of sections of the mouse oesophagus at different developmental stages also showed muscle development (Fig 3.2). The mesenchymal layers (outer layers from the basement membrane of the oesophagus), are only 3-4 layers of cells at E11.5 (Fig 3.2 A). At E13.5, the outer layers of the mesenchymal layers have differentiated and show myofibre morphology although no striations can be found at this stage of development (Fig 3.2 B). At E15.5, two layers in the muscle region are seen (Fig 3.2 C) and are more obvious at E17.5. At E17.5, postnatal day 1 and at adult stages (Fig 3.1 D-I), the two layers of muscle tissue are well developed, we can clearly see the striations in some parts of the muscle layers and in adult, the thickness (~500 μm in average) of the muscle layers are larger than the region of submucosa and epithelium combined (Fig 3.2 H). The endodermal cells (epithelium) actually occupied less space than the mesodermal cells (submucosal mesenchyme and muscle cells) in the oesophagus. The normal muscle layers of the oesophagus starts differentiation as seen in haematoxylin and eosin from E13.5-E15.5, and striated muscle are found from perinatal stages (Fig 3.2 G).

3.4.2 Muscle development

Initially, I determined the timing of muscle markers being expressed in mesenchymal cells in both *in vivo* and *in vitro* culture. Desmin is a marker for both smooth and skeletal muscle and is expressed in early myoblasts (Costa et al., 2004). Desmin expression was found in the myofibres of the adult

oesophagus (Fig 3.7 A-C), but not in the epithelial region (cell layers above the black asterisk in Fig 3.7 B) (the light brown colour is residual non-specific staining). There is strong desmin expression in the outer circular and the inner longitudinal layers of the muscularis regions (*muscularis externa*) (red asterisk in Fig 3.7 B) of the oesophagus, and some expression of desmin in the submucosal region especially at the *muscularis mucosa* (black asterisk in Fig 3.7 B). The epithelium showed no expression of desmin (Fig 3.7 A, B).

In the *in vitro* culture system, there is no desmin expression from the first day. After 9 days of culture, desmin was found strongly expressed in the fibre-like cells of the outer layers (presumably the muscle cells) (Fig 3.7 E, F), but not in the inner epithelial part of the culture. The expression persists until at least 20 days of culture. The change of expression of the desmin marker in the E11.5d *in vitro* model showed the submucosal region of the explant could differentiate and form the muscle layers.

In order to examine whether transdifferentiation of the muscularis region of the oesophagus occurs *in vitro*, markers of both smooth and skeletal muscle were used for immunostaining. A number of different culture periods were examined (1d, 7d and 15d) (Fig 3.8 E-N). On the glass coverslips culture (Fig 3.8 E-I) as well as on the fibronectin coverslips (Fig 3.8 J-N), no expression of either the smooth muscle (smooth muscle myosin kinase – SmMyoK)) or the skeletal (and cardiac) muscle markers (myoglobin) on the first day of culture was found (Fig 3.8 E, J) (The green and red colour in Fig 3.8 E were non-specific staining which did not correspond to the muscle fibrous cellular shapes). The muscle layers of the cultures that grew on either glass or fibronectin coverslips, expresses both smooth (smooth muscle myosin kinase) and skeletal (myoglobin) muscle markers on 15 days cultures (Fig 3.8 H, I, M), although the

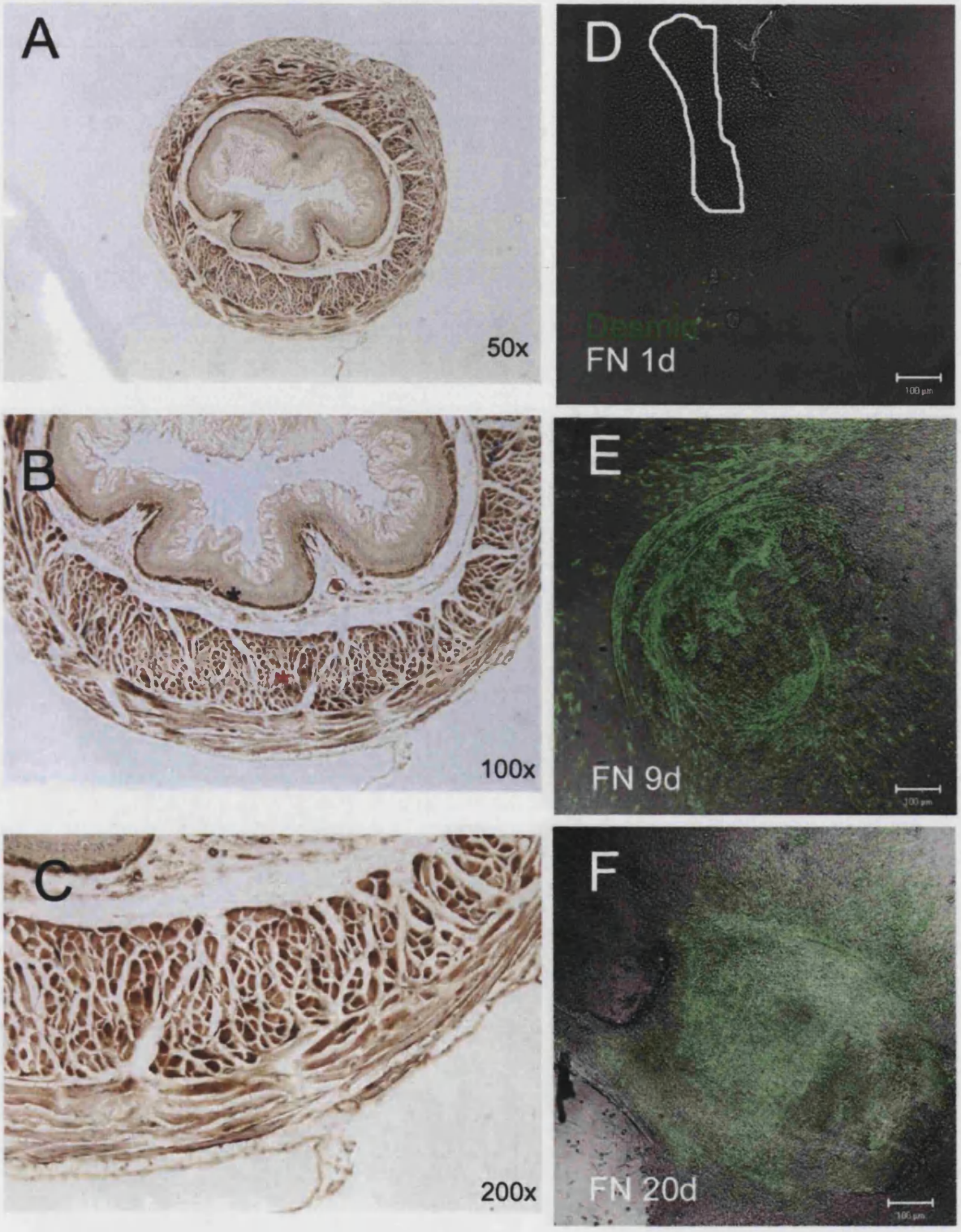

A
50x
B
100x
C
200x
D
FN 1d
E
FN 9d
F
FN 20d

Fig. 3.7 The development of the oesophageal muscular layers in the embryonic culture system – expression of desmin.

Adult mouse transverse section (A-C) was immunostained for desmin, from low to high magnification. Desmin was also stained on the 1 day (D), 9 day (E) and 20 day (F) culture of the E11.5 oesophagus. The expression of desmin, a cytoskeletal marker for both smooth and skeletal muscle (Kablar et al., 2000), is also a marker for early myoblasts. Desmin was found in the adult mouse oesophagus, both in the muscularis mucosa region (black asterisk in B) and the outer circular and inner longitudinal muscularis region (red asterisk in B). On the first day of culture, desmin was not expressed, but was positive at 9 days and 20 days of culture. This change of expression of desmin showed the submucosal region could differentiate into mature muscular layers in the culture system.

A
SMA
100x
B
200x
C
SkMyo
100x
D
200x
E
SmMyoK
Glass, 1d
F
Glass, 7d
G
H
Glass, 15d
I
J
SmMyoK
FN 1d
K
FN 7d
L
M
FN 15d
N

Fig. 3.8 Development of the oesophageal smooth and skeletal muscle layers in the embryonic culture system.

Smooth muscle actin (SMA) (A, B), a marker for the smooth muscle and mesenchymal cells, and skeletal myosin (SkMyo) (C, D), a marker for the skeletal muscle, were immunostained on the adult oesophageal cross sections at low (A, C) and high magnification (B, D). In adult, the expression of the SMA was found both at the muscularis mucosa (black asterisk in A) and the outer circular and inner longitudinal muscularis region (red asterisk in B). The expression profile is the same as desmin (Fig 3.7 A-C). However, the skeletal muscle marker – SkMyo, was only found at the outer circular and inner longitudinal muscularis region (red asterisk in C), but not at the muscularis mucosa (black asterisk in C). Double staining was done for smooth muscle marker (smooth muscle myosin kinase SmMyoK, (Green)) and skeletal and cardiac myocyte marker (myoglobin, (Red)) on the *in vitro* oesophageal cultures (E-N). Embryonic oesophagi were cultured on glass cover slips (E-I), or were cultured on fibronectin (FN) coated cover slips (J-N). Oesophagi were cultured for 1 day (E, J), 7 days (F, G, K, L) or 15 days (H, I, M, N). No expression of either smooth or skeletal muscle markers was found on 1 day of culture, but they were found on 15 days culture on both glass or fibronectin coated cover slips. The skeletal muscle marker was expressed earlier on the glass coverslip than on the fibronectin coated culture (compare F, G, K, L).

expression of the skeletal muscle marker at day 7 cultures on glass (Fig 3.8 F, G) is earlier than on the fibronectin coverslips (Fig 3.8 K, L).

Examples of cells expressing only the SmMyoK (green) or Myoglobin (red) or both (yellow) could be found in both glass (Fig 3.8 I) and fibronectin (Fig 3.8 M) cultures. The coexpression of both smooth and skeletal markers indicates the process of muscle transdifferentiation. Both smooth and skeletal muscle markers are not expressed at day 1, but start to be expressed later during the culture period. This expression profile is the same as the appearance of desmin *in vitro*.

The adult section showed the expression of alpha-smooth muscle actin (SMA) (Fig 3.8 A, B) for smooth muscle and skeletal muscle myosin (FAST) (SkMyo) (Fig 3.8 C, D) for skeletal muscle. SMA expression was identical to the expression pattern of desmin (Fig 3.8 C, D compared with Fig 3.7 B, C), which was positive in both *muscularis externa* (red asterisk) and *muscularis submuscosa* (black asterisk), while skeletal myosin kinase was only positive in *muscularis externa* but not in *muscularis submucosa*. Both smooth (SMA) and skeletal markers (SkMyo) were absent in the epithelium region, although there were stronger background signal in the epithelial region for SMA staining (Fig 3.8 A).

In conclusion, I have used smooth muscle actin and smooth muscle myosin kinase as smooth muscle markers, skeletal muscle myosin and myoglobin for skeletal muscle markers and desmin as a general marker for both smooth and skeletal muscle. The adult oesophagus is used as a positive control for the markers. Interestingly, in the adult, the results from desmin, SMA and SkMyo indicate that the *muscularis submucosa* region contains smooth muscle but not skeletal muscle. I also compared fibronectin coated versus uncoated glass

coverslips for culture and examined the time course for expression of smooth and skeletal muscle markers, in both conditions neither smooth muscle markers (SMA and desmin) nor skeletal muscle markers (SkMyo and desmin) appear on the first day of culture. Instead, skeletal muscular differentiation occurs from 7 days of culture onwards.

3.5 Discussion

The aim of the research described in this chapter was to describe the development of the epithelium and muscle regions in the oesophagus. I needed evidence of changes in morphology and cell-specific markers to gain more understanding of the oesophageal development. I also established an *in vitro* embryonic oesophageal culture system to recapitulate the development of the oesophagus *in vivo*. Both aims were achieved.

Below is a table summarising the results of the epithelial and mesenchymal / muscular expression profile comparing *in vivo* and *in vitro* systems.

Table 3-3 Expression profile of differentiation markers of the oesophagus cultured on fibronectin.

Epithelial and Mesenchymal / Muscular Markers	*In vitro* expression time points (isolated from E11.5)	*In vivo* expression time points
K8 (K18)	day1-day15	E11.5 - ~P5
K14	day5->day20	E15.5 – adult
K10	day5-day20	E17.5 – adult
K4 (K13)	day1-day20	E11.5 – adult
Involucrin	day3-day20	E13.5 – adult
Filagrin	day7-day20	E17.5 – adult
Desmin	day3-day20	adult, not done in embryos
α-Smooth muscle actin	day3-day20	adult, not done in embryos
Smooth muscle myosin kinase	not done	adult, not done in embryos
myoglobin	>day7->day15	adult, not done in embryos
Skeletal myosin	Not done	adult, not done in embryos

3.5.1 Characteristics of the in vitro culture model system

The epithelium and muscle tissue develop in oesophagi isolated from mouse embryos when cultured on fibronectin-coated coverslips. However, the morphology of the *in vitro* oesophagus may differ to the oesophagus *in vivo.*

First, the cultured oesophagus is no longer supported by other organs such as trachea and lung that normally develop around the oesophagus, and the shape of the oesophagus lying on the flat surface also changes to a flattened morphology with hardly any space remaining as lumen (Fig 2.1 C). However, the epithelial cell morphology and the differentiation markers still changes from columnar (red cells in Fig 3.4 A) to stratified (many layers) and squamous-like (flattened) (red cells in Fig 3.4 G).

Second, it is clear that there are mesenchymal cells spreading on the glass coverslip or the extracellular matrix-coated surface. For example, cells that spread around the oesophagus buds are shown in Fig 3.5 F, Fig 3.7 D and Fig 5.1 C. The origin of these fibroblastic-like mesenchymal cells remains elusive but one might postulate they come from serosa, the outer layer of the culture. This is because the epithelial and muscular cells form concentric layers in the cultured buds, in which their organization are usually intactly kept. This lack of coherence in some of the mesenchymal cells in culture is somehow different to the oesophageal development *in vivo.*

Third, the mechanical stress and tension force applied to the oesophagus is different between culture on the solid surface and the normal *in vivo* arrangement. It is known that mechanical forces are crucial to the regulation of cells and tissue morphology (Katsumi et. al., 2004). Extracellular matrix proteins can activate their receptor proteins-integrins to facilitate the focal

adhesion of cells and mechanotransduction of force to the cells. Forces influences cytoskeletal organization, gene expression, and survival and proliferation signals to cells. It is now known that in addition to structural functions, many components of the adhesion apparatus also possess signalling capabilities (Jamora and Fuchs, 2002). The mechanical force and tension applied is apparently very different between oesophagus developed *in vivo* and the whole-mount culture attached on the flat solid surface. The different time points of skeletal muscle marker expression between oesophagi cultured on the glass coverslip and on the fibronectin-coated coverslip (Fig 3.8 G vs L) might be due to specific effects of the different extracellular matrix proteins on the signalling to the focal adhesion complex and thus the rate of inducing cellular differentiation.

3.5.2 Oesophageal stratified squamous epithelium

The main focus of this study is on the differentiation of the oesophageal epithelium. The epithelium of the oesophagus is just simple columnar at E11.5 day. It gradually becomes multilayered and became a fully differentiated stratified squamous tissue after birth (or ~ 15 days culture). The typical stratified squamous markers K10, involucrin, loricrin and filaggrin were found in our culture system accordingly. Simple columnar markers such as K8 and K18 were expressed from E11.5 (1 day culture) but were lost from the basal layer at perinatal stages (~7-9 days culture) and totally disappeared from the epithelium in 2 months (or 20 days culture). On the contrary, K14 the basal layer marker of a stratified squamous tissue becomes expressed around E15.5-E17.5 (5-7 days culture), and gradually the whole epithelium is K14 positive after birth (or after 9-11 days culture). Thus there is a change from

simple columnar to stratified squamous tissue in the oesophageal epithelium during development.

3.5.3 Muscle development and changes in the oesophagus

To study muscle development in the oesophagus, I determined the dramatic changes of morphology of mesenchymal region in the oesophagus during the time points of E11.5d – 2 months old mouse. Within the mesenchymal layer, the muscular region becomes distinct from other fibroblastic cells at about E13.5d, when the mesenchymal region could be seen to divide into the submucosal and muscularis region. The two layers of muscular tissue are seen from E15.5, and striation of the muscle is found from E17.5 in our oesophageal sections. Next, both markers of smooth muscle and skeletal muscle were shown to be expressed in the muscular region of adult oesophagi. Using α-smooth muscle actin and smooth muscle myosin kinase as markers for smooth muscle, myoglobin and skeletal muscle myosin (fast muscle) as markers for skeletal muscle, and desmin as a marker for both smooth and skeletal muscle, the spatial and temporal profile of the muscle specific markers was characterised.

From the results of the changes in expression of the skeletal muscle marker-myoglobin in the oesophagi cultured on glass coverslip (earlier) and fibronectin-coated surface (later) (Fig 3.8 G vs L), one might imagine that the change of extracellular matrix proteins provided near the muscle layers may influence the development of the muscle. The expression of stratified squamous epithelial markers, on the other hand, has less variation between oesophagi grown on different extracellular matrix-coated surfaces (Table 3-2 and data not shown). This might be because the epithelial layers are

influenced by immediate signals from the extracellular matrix proteins of the basal lamina and the submucosa mesenchymal cells, which are in contact with the epithelium of the cultured oesophagus no matter which coverslip coating.

3.5.4 Is there transdifferentiation of oesophageal muscle?

To prove that "transdifferentiation" occurs in cells is to trace the cell lineage before and after the phenotypic and molecular/biochemical change of the cells. In the present study, two conditions I examined, i.e. culturing embryonic oesophagus on glass or fibronectin coated coverslips, muscle cells either expressed markers of smooth muscle specific only, markers of skeletal muscle specific only or cells that contained both markers (undergoing transdifferentiation).

Zhao and Dhoot argue that α-smooth muscle actin (SMA), the smooth muscle marker they used is found in both smooth and skeletal muscles at the foetal stages (Zhao and Dhoot, 2000), and at later stage, SMA became a specific marker to the mesenchymal / smooth muscle but not skeletal muscle. In our system, SMA and smooth muscle myosin kinase were used as smooth muscle markers. It seems unlikely that two markers for smooth muscle are both non-specific at the early stage. With the finding of myoglobin and SmMyoK co-expressing cells (Fig 3.8 I, M) and myoglobin expressing only cells (Fig 3.8 I, N) in the 15 days cultured E11.5 oesophagus, the specificity of SmMyoK as a smooth muscle marker is established.

For the lineage tracing studies using smooth muscle promoter driven Cre and GFP (Rishniw et al., 2003), we might also argue that in their transgenic mice, the Cre protein driven by the smooth muscle myosin heavy chain promoter needs time to accumulate for the Cre recombinase to function, and LacZ will

not express as early as the real smMHC. This might result in the Cre induced LacZ expressed at a later time than the normal expression hence missing the transdifferentiation event (cf. Fig 1a and 1b ED15 GFP and LacZ results in Rishniw et al., 2003). Our muscle marker staining results also showed comparable time points in the changes of the skeletal markers in the *in vitro* culture to another paper on this topic (Kablar et al., 2000).

The findings that some cells express both smooth and skeletal muscle markers in the oesophageal cultures, suggests that the origin of the skeletal muscle cells is very probably from the smooth muscle cells. This may constitue an example of transdifferentiation. However, there are also cases such as insulin and glucagon co-expressing cells found during embryogensis are actually not the precursors of these two cell types in the adult (Herrera, 2002), and we still cannot rule out the possibility that there are other types of skeletal muscle precursor cells that actually outgrow and replace some smooth muscle cells in parts of the oesophagus.

3.5.5 Conclusions and directions

In summary, the morphological change of the mouse oesophagus in the muscle and epithelium regions was shown from E11.5 to adult. Specific markers were used to confirm the changes of both columnar to stratified squamous epithelium and smooth to skeletal muscle during development of the oesophagus.

What is the mechanism of the change from simple columnar to stratified squamous epithelium? Is it specified before the foregut endoderm separates into trachea and oesophagus? If this is the case, there must be a difference in the ventral and dorsal patterning in the undivided foregut, but then we must go

to an earlier time point (E9) to verify the differential gene expression or the signalling mechanisms. If the stratification process is specified after the oesophagus bifurcates from the foregut, then there must be a change in either signalling or gene expression during the time points we are studying. Also, what are the candidate genes involved in this process? Is there a correlation (in both genes and signals) between the changes from simple columnar to stratified squamous during development and the stratified squamous to simple columnar metaplasia? Maybe there is a universal mechanism in all types of stratified squamous metaplasia that happens in tissues like breast epithelium, cervix / vagina epithelium or bronchial regions of the lung.

To begin to answer these questions, I certainly would like to verify whether the epithelial switch is in fact a transdifferentiation event. In the next chapter, I will start by asking what is the source of the K14 cells arising from the basal layer.

Chapter 4 Cell fate and origin of the developing oesophageal epithelium

4.1 Introduction

4.1.1 Is the conversion of columnar to stratified squamous epithelium an example of transdifferentiation?

In Chapter 3, I demonstrated that the phenotype of the oesophageal epithelium changed from simple columnar to stratified squamous both *in vivo* and *in vitro.* The change of oesophagus morphology was shown (in vivo) in Fig 3.2, markers of columnar markers to stratified squamous markers were shown in Fig 3.3 (in vivo), and Fig 3.4 (in vitro) and terminal differentiation markers of a stratified squamous tissue were shown in Fig 3.5-3.6. The aim of the present chapter is to extend the results presented in Chapter 3 to investigate whether the normal development of the oesophagus could be attributed as a transdifferentiation event. According to Eguchi and Kodama, in order for a process to be considered as transdifferentiation, a number of criteria must be fulfilled (Eguchi and Kodama, 1993; Eguchi, 1995). The first is that the phenotype of the cells at the start and the end of the conversion must be clearly defined. The second is a clear demonstration of the ancestral-descendent relationship. Put simply; we wish to understand the cellular origin of the basal stratified squamous cells. To address this problem, I investigated three lines of evidence: (1) Whether there were co-expression of columnar and stratified squamous markers in the same cells (both *in vivo* and *in vitro*) during the time period when stratified squamous cells were appearing. For this purpose, representative markers of the two cell types were examined in the oesophagus. (2) DiI labelling was employed to trace the cell fate of the

basal epithelial cells and (3) using the stratified squamous basal cell specific-K14 promoter driving a GFP reporter, the cell lineage was determined.

4.1.2 DiI cell lineage tracing

1,1'-dialkyl-3,3,3',3'-tetramethylindocarbocyanine (CN*dil*) dyes (N = 12, 18, and 22) or DiI is a lipophilic cell tracer composed of a long-chain dialkylcarbocyanine, which emits at 565 nm, and gives an orange-red fluorescent colour (Packard and Wolf, 1985). DiI has been widely used for tracing live cells in tissues as it is readily absorbed in the cell membrane. DiI apparently does not affect the viability and developmental or physiological properties of cells that take it up. For example, motorneurons labelled with DiI can be traced for up to 4 weeks *in vitro* and up to a year *in vivo* (Honig and Hume, 1986; Honig and Hume, 1989). In addition to cell tracing, lipophilic carbocyanines have many other applications such as the detection of cell-cell fusion and adhesion, tracing cell migration during development and after transplantation, cytotoxicity assays and labelling of lipoproteins. In the present study, I used E13.5d oesophageal cultures for DiI cell labelling. This time point was chosen as the epithelial cells are still columnar. Assuming DiI can remain incorporated into the cells for a few days, I should be able to show the presence of DiI and K14 in the same cells. I will present evidence for DII labelling of stratified squamous cells suggesting direct conversion from columnar cells.

4.1.3 Cell fate analysis in the developing oesophagus

I was also interested in the cell fate of the columnar and stratified squamous cells in the developing oesophagus. If the stratified squamous cells do not

arise due to direct transdifferentiation, but via replacement of the columnar cells by the squamous lining of the oesophagus, I should then see an increase in the rate of cell death of columnar cells and an increase in the proliferation of stratified squamous cells. These results would tell us whether there is an overgrowth of the stratified squamous progenitor cell population.

4.1.4 Apoptosis and development

Programmed cell death, also known as apoptosis, can be generally categorised into two main pathways: the death-receptor pathway and the mitochondrial pathway (Hengartner, 2000). In both situations, the process of programmed cell death is through a cascade of proteolytic reactions in which caspases are the central executioners. Chemical caspase inhibitors, such as zVADfmk, normally inhibit apoptosis, although, new evidence shows there are examples of caspase-independent programmed cell death (Abraham and Shaham, 2004). Caspases have been found to work not only as an apoptosis executioner, but have other roles such as regulators of cell differentiation (Abraham and Shaham, 2004). Apoptosis is also important for the development of an organism (Meier et al., 2000), for example the degeneration of the temporary functioning pronephric kidney tubules in mammals and the regression of the tadpole tail during metamorphosis. Both these processes depend on programmed cell death to remove the parts that are no longer necessary for the developing animal. This could be a common theme for organisms to remove specific cells or tissue. I will address the question of whether the columnar cells were lost by apoptosis.

4.1.5 Studies of proliferating cells in the human oesophagus

Studies in the mouse oesophagus have shown that the epithelial layer is initially composed of cuboidal ciliated cells which become replaced by squamous cells from about E17 (Raymond et al., 1991). By E17, ^{3}HTdR labelling is confined to the basal layer and the suprabasal cells of the mouse squamous epithelium without the proliferating capacity became keratinised 8 days after birth. Ki67, a proliferation marker for cells actively going through cell cycle (Endl and Gerdes, 2000), was used in the present study to find the location of the dividing cells in the oesophagus culture.

4.1.6 Metaplasia with intervening cell division

For most examples of metaplasia, we would imagine that the cells undergoing phenotypic changes must go through a number of cell divisions (Beresford, 1990). The reason for this is because metaplasia in the adult is a rare event, and it is very difficult to imagine how "all" cells in a large area of a metaplastic focus will directly switch to the phenotype of another. Therefore, the more likely possibility is that a small population of cells in tissue A has changed phenotype to progenitor-like cells of tissue B, which then proliferate to populate the whole metaplastic area. Dedifferentiation may be a necessary intermediate in metaplasia before the direct switch and cell division begins, but this needs to be studied. However, metaplasia usually requires a long period of time to develop. It is technically awkward to address the question of cell division *in vivo* and an alternative is to utilise techniques employed by stem cell biologists. For example, proliferating cells can be visualised in a tissue by injection of BrdU to trace the cells that have incorporated the label (Tumbar et al., 2004). The long-retaining labelled cells that have been pulsed once for the BrdU and chased for a long period of time (e.g. 6 months) may be seen as a stem cell.

An *in vitro* model system becomes more useful under these circumstances. If we can provide a reliable system in culture that represents a model of how metaplasia occurs, details of the cellular and molecular pathways that are involved in metaplasia / transdifferentiation can then be determined.

In this chapter, I will address (a) the cell lineage of the switch in epithelial phenotype and (b) whether cellular division and cell death plays a role in the switch.

4.2 Co-expression of columnar and stratified squamous markers

4.2.1 In vivo and in vitro evidence

I employed a number of different approaches to determine if stratified squamous cells arise from columnar cells. First, I examined the K8 and K14 expression *in vivo* and *in vitro*. From previous results (Chapter 3, Fig 3.5 K, L), we know that the stratified squamous basal layer marker appears around E17.5, and the columnar marker starts to disappear around E15.5. I used two colour DAB immunostaining for K8 and K14 on sections from E18.5 embryos in order to find whether the keratins are co-expressed at the time point when the markers are just switching from columnar to stratified squamous at the basal layer. The oesophagus (Fig 4.1 A-F), epidermis (Fig 4.1 G) and bronchial region (Fig 4.1 H) show sharply contrasting staining patterns with K8 and K14 antibodies. The epidermal cells stain only for K14, presumably because the epidermis has turned into a stratified squamous tissue earlier than the oesophagus (Fig 4.1 G). On the contrary, the bronchial epithelium which is pseudo-stratified, stained only for K8 (Fig 4.1 H). In the oesophagus we see the expression of both K8 and K14 markers in two examples given here (Fig 4.1 A-C and D-F). While the K8 is predominantly found in suprabasal cells, some black granules may also be seen in basal cells that are expressing K14 (arrows in Fig 4.1 B, C, E, F) (co-expressing cells were found in 3/3 cases. The number of coexpressing cells are 21, 28 and 16 on the 3 sections). Three consecutive sections of an E17.5 oesophagus were also stained for K14 (Fig 4.1 D) or K8 (Fig 4.1 E) alone and in combination (Fig 4.1 F). I found that K14 stains the basal layer (Fig 4.1 D). Although K8 is predominantly found in suprabasal cells, there is also much weaker staining at the basal layers (Fig 4.1 E). This is masked in the dual-stained section (Fig 4.1 F), but when

adjacent sections are singly stained it is clear that some cells do express both K8 and K14. A transitional expression of both markers would be expected if stratified epithelium does indeed arise from the columnar cells by transdifferentiation.

From previous *in vitro* staining results (Fig 3.3 C), we know that K14 cells starts to express in the oesophagus culture from around day 5 (co-expressing cells were found in 4/5 of 5d cultures). The second proof of cell lineage is to find whether any cells simultaneously expressed both markers in the 5 day cultured E11.5 oesophagus. Again, this showed that the K14 cells start to appear in the basal layer while some K8 expression still persists in the basal layer. Some emerging cells were found to have both markers (arrows in Fig 4.1 I, this is an enlargement of Fig 3.3 C).

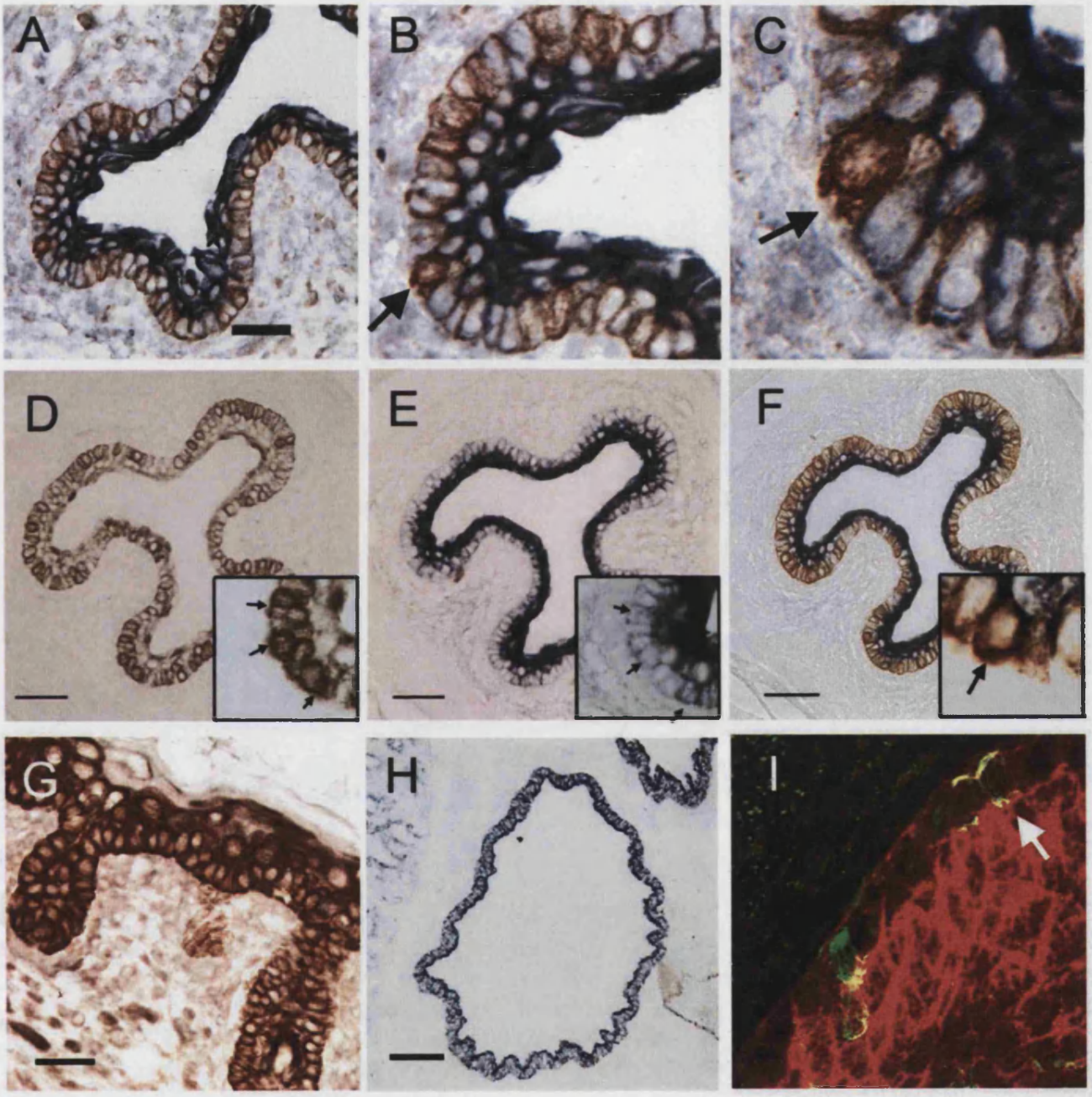

A
B
C
D
E
F
G
H
I

Fig 4.1 Expression of K8 and K14 in oesophageal and other epithelia.

Dual immunohistochemical staining for K14 (brown) and K8 (black) in E18.5 oesophagus (A-C), E17.5 oesophagus (D-F), epidermis (G) and bronchial epithelium (H). (B) (1.3 x of (A)) and (C) (1.69 x of (A)) show higher magnification of the area in (A) where there is a co-stained cell (arrow). Example of single and dual staining for K14 (brown) and K8 (black) in consecutive sections of E17.5 oesophagus were shown in (D-F). K14 (D), K8 (E) and K8 and K14 (F). Black arrows indicate cells expressing both cytokeratins. At the early stage of the conversion, some oesophageal cells express both K14 and K8, while the epidermis is only positive for K14 (G) and the bronchial epithelium is only positive for K8 (H). Confocal images of dual staining for K14 (green, FITC) and K8 (red, TR) in 5 day E11.5 oesophageal culture (I) (a further 10x enlargement of Fig 3.3 C). The arrow points to some cells starting to express K14 in the K8 positive cells. Scale bars are 80 μm in (D, E, F) and 200 μm in (A, G, H).

4.3 Lineage analysis using DiI and perdurance of green fluorescence protein

4.3.1 DiI lineage tracing

To complement the results of the co-expression of the columnar and stratified squamous markers, I have examined the lineage using cell tracing techniques during the epithelium conversion period. Initially, a segment of epithelial cells lining the oesophageal lumen was labelled with a lipophilic acetylated red dye - DiI. Injecting DiI into the lumen of an E13.5 oesophagus will result in the dye being quickly incorporated into the cell membrane of the lining epithelium. In theory, with cell division, the concentration of DiI in each daughter cell will be diluted. When tracing the cell fate of the K8 positive cells lining the lumen, the earliest feasible time point for micro-injection of cell lineage tracing markers is at E13.5. Earlier than this time point, there is virtually no lumen in the oesophagus or the space of the lumen is just about one cell thick (see Fig 3.2 A and 3.5 A). Presumably, this restriction is because the oesophagus has not yet undergone the vacuolization process, and there is still constriction of the lumen (DeNardi and Riddell, 1991).

From previous results (Fig 3.5 B, J), we know that all cells of E13.5 oesophageal epithelium are K8 positive and K14 negative. Assuming cells in the E13.5d oesophageal epithelium incorporate DiI, then after the basal cells switch phenotype, there should be some cells that contain label and express K14. This would provide additional evidence that the K14 positive cells were formed from K8 positive cells of the E13.5 oesophagus. First, we show how the E13.5 oesophageal epithelial cells take up DiI. Fig 4.2 A shows a live image of an E13.5 oesophagus lumen cultured 24 hrs after injection with the red DiI. DiI labels the whole segment of epithelial cells in the region where injected. I also

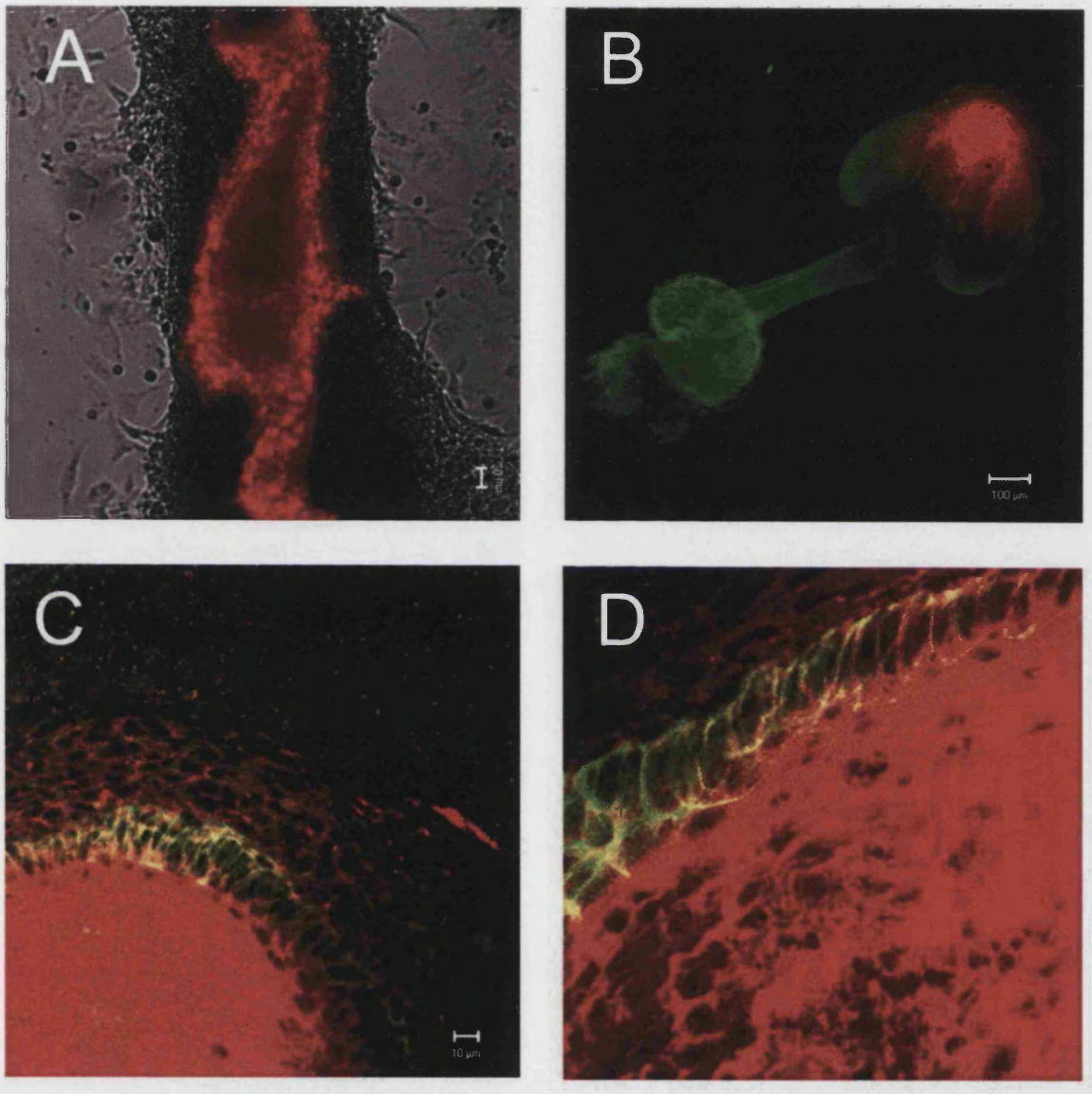

A
B
C
D

Fig 4.2 DiI cell lineage tracing of oesophageal epithelium.

Live image of an E13.5 oesophagus injected with DiI and cultured for 24 hrs (A) and an example of tracing an E13.5 oesophagus cultured for 9 days after DiI injection (B-D). Cells that take up DiI were K8 positive at the time of injection, and during the culture period, the epithelial cells that were red start to express the basal stratified squamous marker K14. K14 was stained after the DiI injected E13.5 oesophagus was cultured for 9 days. Yellow cells in the epithelium (C, D) showed the K14 cells (green, FITC) were originally from the cells that contained DiI (red).

injected an E13.5 d oesophagus and left it for 9 days in culture showed in Fig 4.2 B-D. This should be sufficient time to allow conversion from columnar to stratified squamous epithelium. After fixation, the culture was immunostained with K14 to examine whether the stratified squamous cells are DiI labelled. In Fig 4.2 C and D, we see cells that are labelled with DiI are stained with the K14 antibody. In higher magnification, we can distinguish the region where DiI is less intense, the green (K14) and red (DiI) are found together in the basal layer. This suggests the K14 cells do come from DiI labelled cells, although in this case, we see that some layers of cells beneath the basal layer are also labelled faint red, which is probably due to diffusion of DiI into the mesenchymal layers from the lumen. Similar results were also found in E13.5 day injected cultured for 5 days (not shown).

4.3.2 Activation of K14 promoter in K8 expressing cells

Although the DiI labelling experiment is suggestive of a columnar origin for the K14 positive cells, I could not be absolutely certain of the cell origin of the K14 expressing cells from the DiI tracing experiment due to the fact that some non-epithelial cells may also be labelled with DiI. Another approach I used is to construct a reporter plasmid to perform cell lineage tracing. This reporter consists of the stratified squamous *K14* promoter driving the expression of GFP with a nuclear localizing signal. The plasmid was introduced into the epithelium by electroporation at a time when the cells are just switching from the columnar phenotype to stratified squamous. Oesophageal segments from E15.5 embryos were dissected and then electroporated with the plasmid. The oesophagus was cultured for 24 hrs and then co-stained for GFP and the columnar K8 marker (diagram to explain shown in Fig 4.3 A). In those cells that

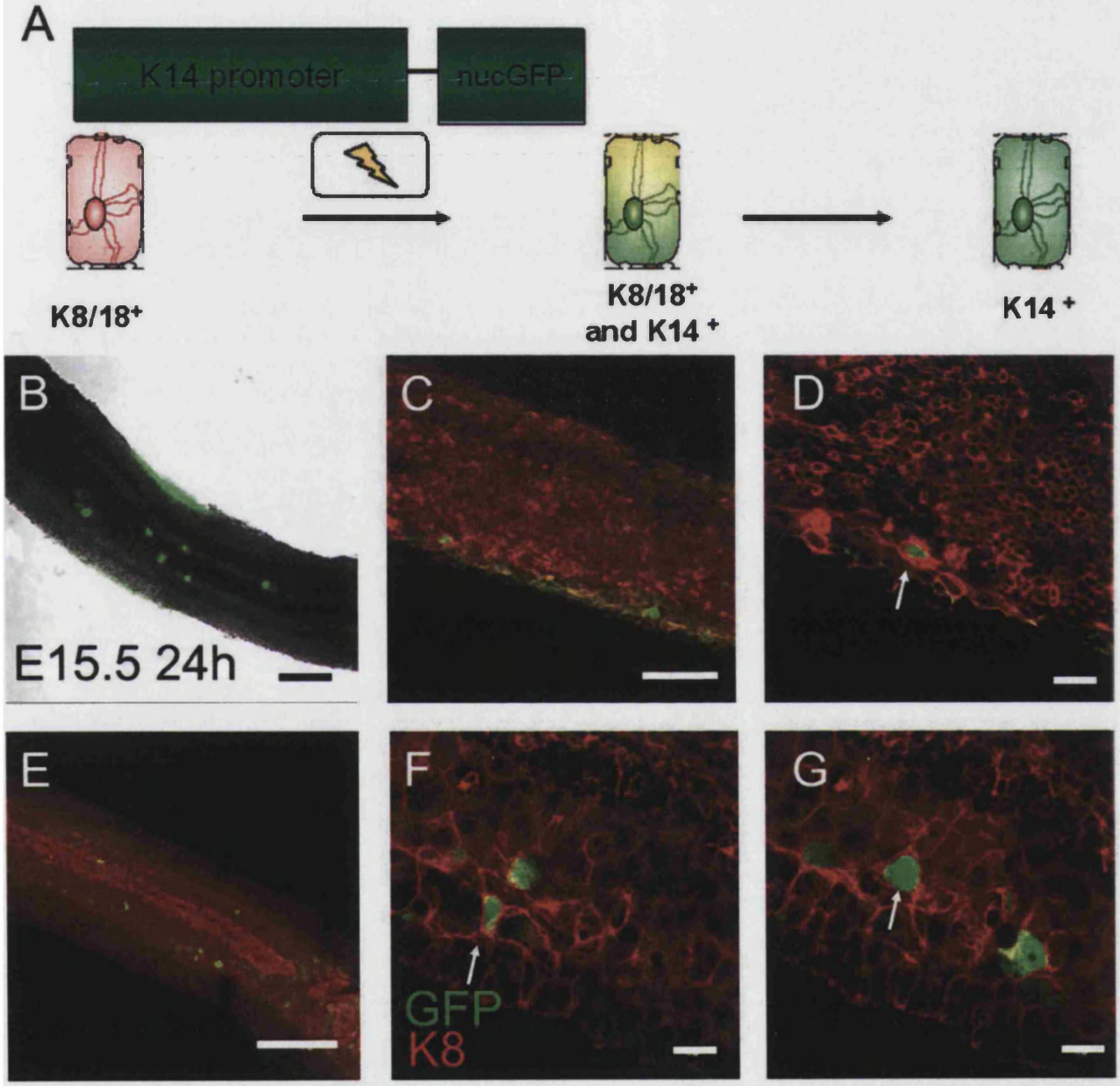

A
K14 promoter
nucGFP
K8/18+
K8/18+
and K14 +
K14 +
B
E15.5 24h
C
D
E
F
GFP
K8
G

Fig 4.3 Activation of *K14* promoter in K8 expressing cells.

(A) Diagram showing the theoretical basis of the lineage experiment. A plasmid containing the stratified squamous *K14* promoter driving GFP (with a nuclear localisation signal) expressed during the period when the columnar cells are converting to stratified squamous cells. Cells that are becoming stratified squamous should have an active *K14* promoter and therefore possess a green nucleus. Assuming these are starting as columnar cells, the cells should co-express with K8 (shown here in red), and appear as yellow. Two examples are given. (B-D) and (E-G) show staining of the oesophagus 24 hrs after electroporation at stage E15.5. Some cells express both the K8 columnar marker and nuclear GFP (arrows in D,F,G), suggesting the origin of the stratified squamous tissue is from the columnar cells, while other GFP positive cells do not stain red suggesting they have already shut off K8 expression. Scale bars are 10 μm in (D, F, G), 100 μm in (B, C, E).

start to express the *K14* promoter (as judged by GFP expression), at least some cells still express the K8 columnar marker. In the five E15.5 oesophagi injected with the *K14-GFP* construct, four samples showed cells with green nuclei. The numbers of green nuclei were 17, 13, 12, 9 and 0 in the five samples examined, and the number of cells with green nuclei co-stained with K8 (red) were 2, 5, 1, 0 and 0, respectively. Two cultures are shown in Fig 4.3 B-G; and in both, several basal cells express GFP and K8 in the same cell (arrows). There are also some GFP-positive cells that do not express K8, but this is not surprising because during this period cells are shutting off K8 and other genes responsible for the columnar phenotype, so it is likely that some of the cells acquiring and expressing the *K14-GFP* plasmid will already have done so.

In order to establish the fidelity of the expression of the K14 reporter construct, it was also electroporated into the epithelial cells of the stomach, small intestine and tracheal explants from E11.5 embryos and cultured under the same conditions as the oesophagus (Fig 4.4 A-D). These epithelia do not normally express K14 and indeed GFP was not expressed. Under the same conditions, GFP controlled by the CMV promoter was expressed (Fig 4.4 E-G), demonstrating the validity of the electroporation method. Experiments using the *K14* promoter reporter construct transfected into various K14 positive and negative cell lines also provided additional proof of the specificity of the promoter (see Chapter 6, Fig 6.6).

In summary, from the three experiments: (a) some cells co-express K8 and K14 in the oesophageal basal layer both *in vivo* and *in vitro*, (b) DiI tracing of the basal layer K8 cells shows that they later express K14, and (c) the *K14* promoter is activated in some cells still expressing K8. The combination of

results suggests that the basal cells of the squamous epithelium are derived directly from the columnar cells by transdifferentiation.

A
E11.5 Stom&Sml
+ pK14-nucGFP 24h
B
Stom
GFP
K8
C
Trachea/lung buds
GFP
K8
D
Sml
GFP
K8
E
E11.5 Stom&Sml
+ pCMV-GFP 24h
F
Stom
GFP
K8
G
Sml
GFP
K8

Fig 4.4 Specificity of the *K14* promoter.

The *K14* reporter construct was electroporated into tissue that does not normally express the stratified squamous epithelium markers. The same culturing condition for examining the *K14* promoter expression after electroporation in the oesophagus was used for these negative controls. Cultures of E11.5d stomach, small intestine and trachea do not express *K14-GFP* 24 hrs after electroporation (A-D), although *CMV-GFP* is expressed (E-G).

4.4 Cell death during development of the oesophagus

4.4.1 FACS analysis of single cell suspensions

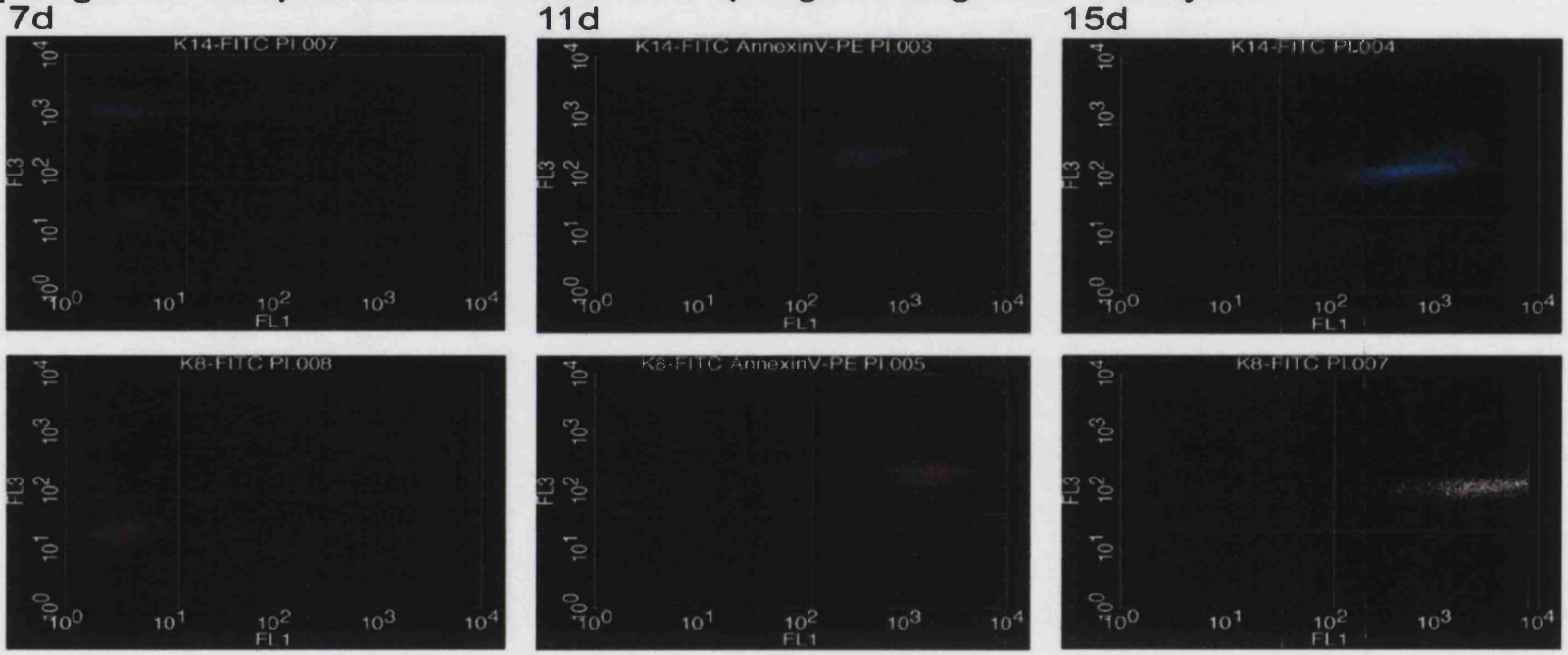
Assuming the conversion of columnar to stratified squamous epithelium depended on a selective overgrowth of columnar by squamous cells; we should expect the rate of cell death to be greater among the columnar cells during the transition period. To investigate this possibility, I quantified the proportion of dead cells in the K8-positive and K14-positive cell populations during the course of the culture period. The oesophageal cultures were trypsinised, and the cells resuspended as a single cell suspension. They were stained with propidium iodide (PI) (staining DNA) and either K8 or 14 antibody and then analysed by flow cytometry. K14 or K8 positive cells are shown as upper-right and lower-right quadrant combined (Fig 4.5 A). Because of the apoptotic cell DNA being fragmented or the DNA fragments appeared in the necrotic cell debris, the Sub-G_0 cells (defined as PI-low in (Fig 4.5 B)) were stained with less PI and were counted as dead cells. This is counted as the percentage of cells from the lower-left and lower-right quadrant combined (Fig 4.5 A) (raw data tabulated in Appendix A.2). The proportion of dead cells in K14 or K8 cells was from each keratin and PI double stained results and calculated as follows:

(percentage of lower-right quadrant) x (percentage of upper and lower-right quadrant combined)$^{-1}$

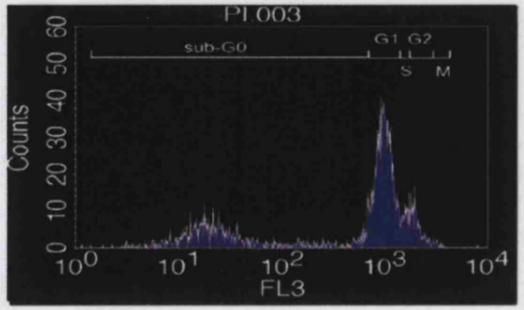
The results show that the proportion of dead cells in the K8 population is about the same as in the K14 population at all three time points examined: 7 days, 11 days and 15 days (Fig 4.5 C). The reason for examining these time points is because this is the period of time when we start to see loss of columnar cells in

the basal then the suprabasal layers of the oesophagus. When expressed as a proportion of particles, the dead cells appear rather more numerous (~50%) than on the intensity profile, this might be because the fraction also includes small subcellular debris which might be caused by pipetting when making single cell suspension. From the FACS analysis, we find slightly more K14 cells than K8 cells are dying using FACS analysis in all three time points (Fig 4.5 C) but the difference is not significant. This shows that the K8 positive cells do not die at a greater rate than the K14 cells.

A Single cell suspension of cultured oesophagus using FACS analysis



B



C

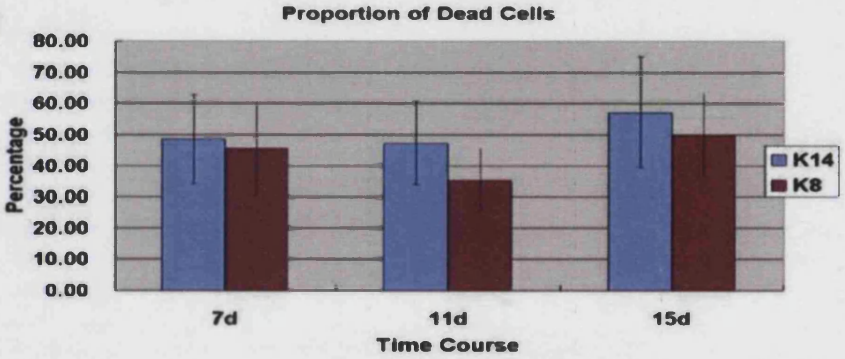


Fig 4.5 Determination of the dead cell proportion in the K8 and K14 cells during oesophagus development.

Intracellular staining of K14 / K8 and PI were carried out on a cell suspension as described in the Material and Methods (Section 2.8) and run through FACS to estimate the dead cell proportion of different cell types after 7 days, 11 days and 15 days of culture. A typical result from each of the time points is shown in (A). A histogram of PI single staining is shown in (B) and the Sub-G0 cells (defined as PI-low in (B)) are counted as dead cells, which is the percentage of cells from the lower-left and lower-right quadrant combined in (A). The proportion of dead cells does not significantly differ from columnar and stratified squamous basal cells in three time points observed (C). Experiments were performed in quadruplicate and the error bar in (C) represents the standard error. Raw data were tabulated in the Appendix A.2.

4.5 Effect of inhibition of apoptosis in the developing oesophageal culture

4.5.1 General caspase inhibitor – zVADfmk

If the switch of cell phenotype were really due to preferential death of one population and resultant overgrowth by the other, then an inhibition of cell death should arrest or delay the process. For this purpose I utilised a general inhibitor of all caspases - zVADfmk. As DMSO was used for dissolving the zVADfmk, 0.1% DMSO was added to control cultures (Fig 4.6 A, B, E, F). To check the effect of zVADfmk on the oesophageal cultures, I added ethidium homodimer to stain the dead cells. The results showed a dramatic reduction in the number of dead cells stained with the red ethidium homodimer, especially in the epithelial region of the E11.5 explants cultured for 7 days (Fig 4.6 C vs A and Fig 4.6 G vs E). To examine the effect on the basal layer transition, I started treating cultures with 40 μM of zVADfmk after 24hrs of culture and continued until day 7. As shown in Fig 4.6 B and D, the disappearance of the K8 cells is similar in the DMSO control treatment and the zVADfmk treated cultures. In fact, comparing Fig 4.6 B and D, the level of K14 in the basal layer of the treated culture exceeds that in the untreated culture, which is the reverse of what would be expected if there is K14 cells outgrow to replace dead K8 cells. I also treated the cultures with zVADfmk from day 8 of culture until day 20, which is the period of loss of K8 cells from the suprabasal layers. In this case too, there was no difference in the rate of loss of K8 cells (Fig 4.6 F vs H).

Both the results of the flow cytometry analysis and the lack of effect of the caspase inhibitor show that progammed cell death is not a significant mechanism by which the columnar cell phenotype is lost in the oesophageal

epithelium.

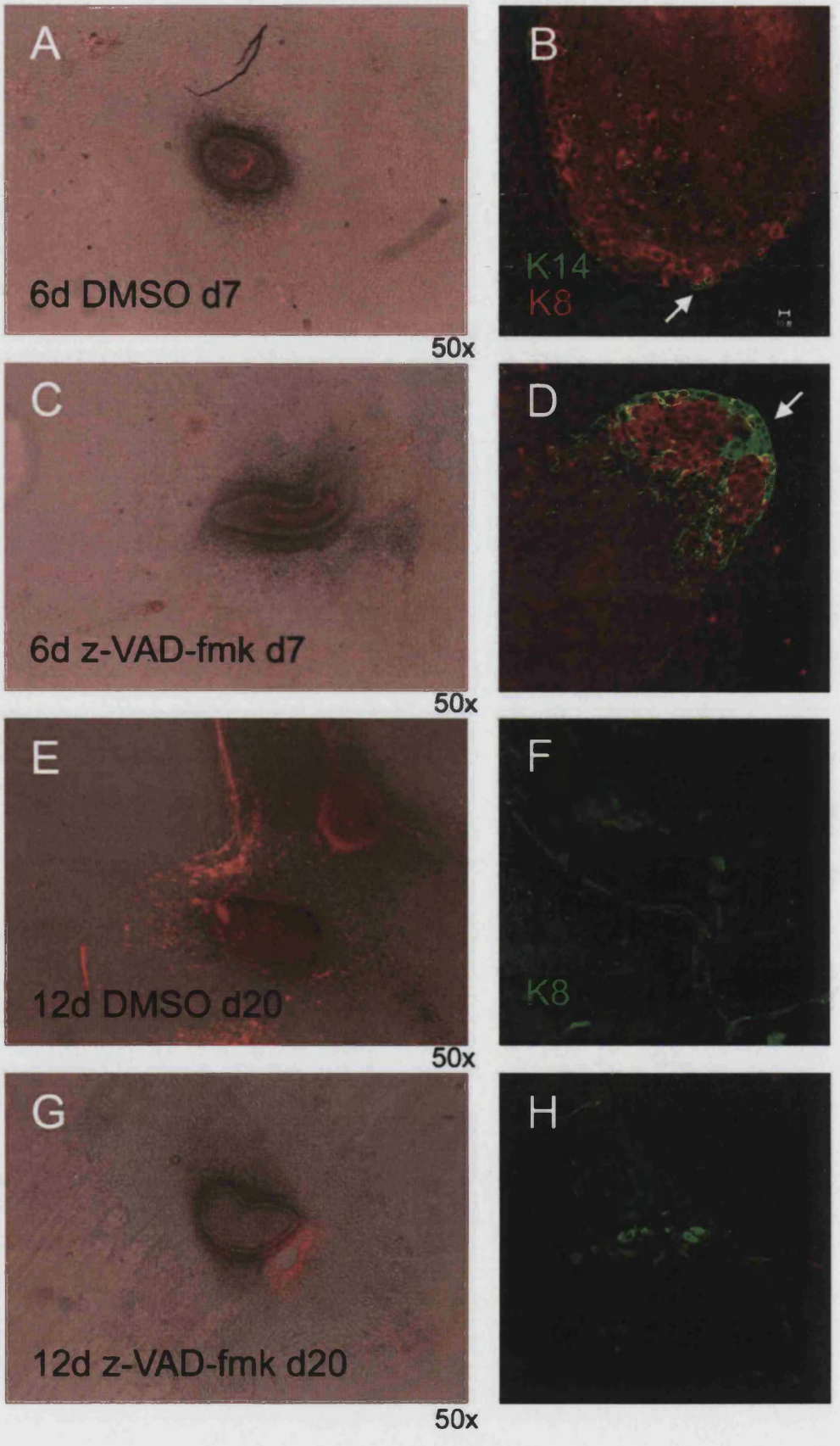

A
6d DMSO d7
50x
B
K14
K8
C
6d z-VAD-fmk d7
50x
D
E
12d DMSO d20
50x
F
K8
G
12d z-VAD-fmk d20
50x
H

Fig 4.6 Loss of columnar cells does not occur by programmed cell death.

To understand whether apoptosis has a role in the loss of K8 cells at the basal layer, E11.5 oesophagus organs were cultured for 7 days (A-D) and treated with 0.1% DMSO control (A, B) or with 40 μM zVADfmk (C, D) for 6 days. To examine if apoptosis is the mechanism for loss of K8 in the suprabasal layers, the organs were cultured for 20 days (E-H) and treated with 0.1% DMSO control (E, F) or with 40 μM zVADfmk (G, H) for 12 days. Afterwards, oesophageal cultures were incubated with ethidium homodimer for 30 min to label the dead cells with red fluorescence (A, C, E, G). K8 (red, TR) and K14 (green, FITC) were used to stain the cultures after 7 days (B, D) or K8 (green, FITC) only staining cultures for 20 days (F, H). Many dead cells are clearly visible under control conditions (A, E) but very few in the cultures treated with the caspase inhibitor (C, G). The red fluorescence in (C) is auto-fluorescence, whereas true dead cells appear as rounded red spots, of which a few are visible in the mesenchyme. (B) and (D) show that preventing cell death does not affect the disappearance of K8 cells from the basal layer after 7 days of culture, (F) and (H) show the same result for suprabasal layers after 20 days of culture.

4.6 Cell proliferation in the developing oesophagus

4.6.1 Ki67 expression in cultured embryonic oesophagus

If the switch in cell phenotype occurred by selective overgrowth of K14 positive cells, then it could not occur in the absence of cell division. Alternatively, if the mechanism depends on transdifferentiation with an obligatory cell division step, it could still not occur. To identify proliferating cells I immunostained for Ki67, a nuclear protein expressed in cells undergoing active phases of the cell cycle (Endl and Gerdes, 2000). The proliferative cells in mature stratified squamous tissues (e.g. skin) are located in the basal layer (Fuchs and Raghavan, 2002). In the early embryonic oesophagus, the epithelium showed high proliferative capacity throughout (Fig 4.7 A, B). When the culture differentiates into multiple layers, the Ki67 positive cells are, as expected, localised exclusively to the basal layer of the epithelium (Fig 4.7 J-L) as well as in some of the mesenchymal cells of the culture, but not in the K8 positive cells any longer (Fig 4.7 C-F). Interestingly, we find the proportion of K14 cells that are Ki67 negative at the time when K14 starts to appear - day 5 culture (9 out of 18 - results were obtained from Fig 4.7 H, I, high magnification images from both ends of the oesophageal culture of Fig 4.7 G), is slightly higher than day 7 culture (15 out of 43) (from Fig 4.7 K) and day 11 culture (14 out of 35) (from Fig 4.7 L), when Ki67 is restricted to just K14-expressing cells. The fact that there are less proliferating K14 cells when they just appear suggests the transition may not require cell division at the start, but the basal cells after the switch became highly proliferative. This hypothesis could be tested if I can inhibit the cell division before the switch has occurred.

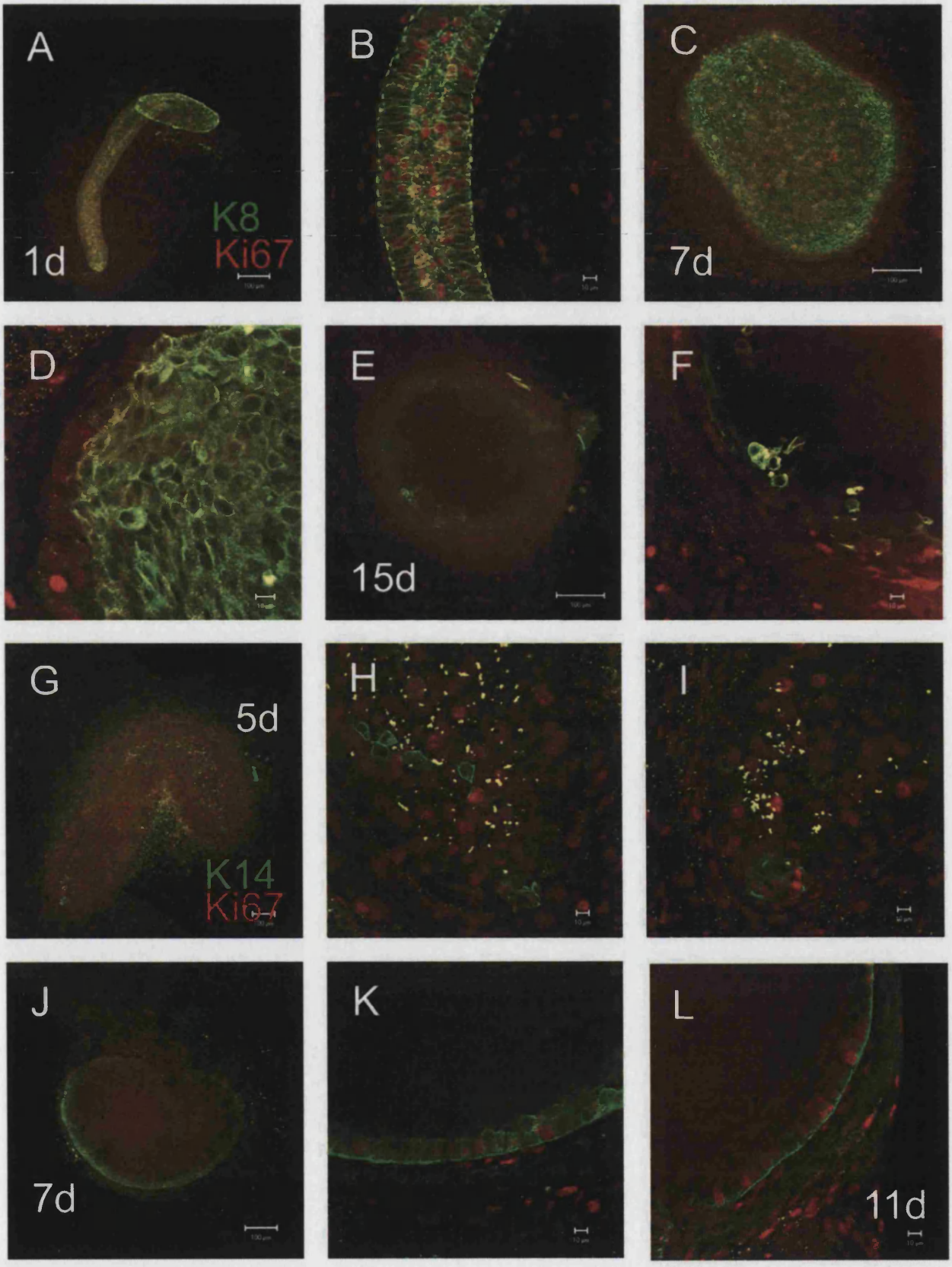

A
K8
Ki67
1d
B
C
7d
D
E
15d
F
G
5d
K14
Ki67
H
I
J
7d
K
L
11d

Fig 4.7 Location of proliferating cells in the oesophageal culture.

Oesophageal bud cultures were stained for K8 (green, FITC) and Ki67 (red, TR) in (A-F); or K14 (green, FITC) and Ki67 (red, TR) in (G-L). 1 day culture result is shown in (A, B), 5 days in (G, H, I), 7 days in (C, D, J, K) and 11 days in (L) and 15 days in (E, F). At the beginning of the culture, many of the epithelial cells in the columnar marker K8 positive layer of oesophageal cultures expressed the Ki67 proliferation marker. At 5 days of culture, when K14 positive cells start to appear, some of the stratified squamous cells are Ki67 positive. After 7 days of culture, only the basal layer K14 positive cells but not the suprabasal K8 positive cells showed proliferative capacity (compare C, D with J, K and F with L). Many of the mesenchymal cells at different stages show Ki67 positive cells.

4.7 Effect of a proliferation inhibitor on the developing oesophageal culture

4.7.1 Mitomycin C does not affect the switch of the epithelium in the oesophagus

To examine the requirement for cell division, I added mitomycin C, a DNA cross linking reagent that blocks cells from entering the cell cycle (Kubilus et al., 1981), to the E11.5d oesophageal cultures. At day 4, the cultures were treated with 1, 5 or 10 µg/ml mitomycin C for 24 hours and then cultured for a further 5 days (without mitomycin C) until day 9. During the 5-9 day culture period, the basal epithelial layer normally completes the change from columnar to stratified squamous. We find the mitomycin C-treated cultures much smaller than the controls. The epithelial area of the 5 µg/ml mitomycin C treated culture was 0.22-x of the PBSA control, and the area of 10 µg/ml mitomycin C treated culture was 0.1-x of the control (Fig 4.8 A, D, G, J). In addition to the differences in area, Ki67 expression is rarely found in the 5 µg/ml treatment and is completely absent in the 10 µg/ml treated cultures (Fig 4.8 I, L). However, a complete basal layer of K14 positive cells continues to appear in the epithelium treated with mitomycin C. This indicates that the switch from simple columnar to stratified squamous does not require cell proliferation.

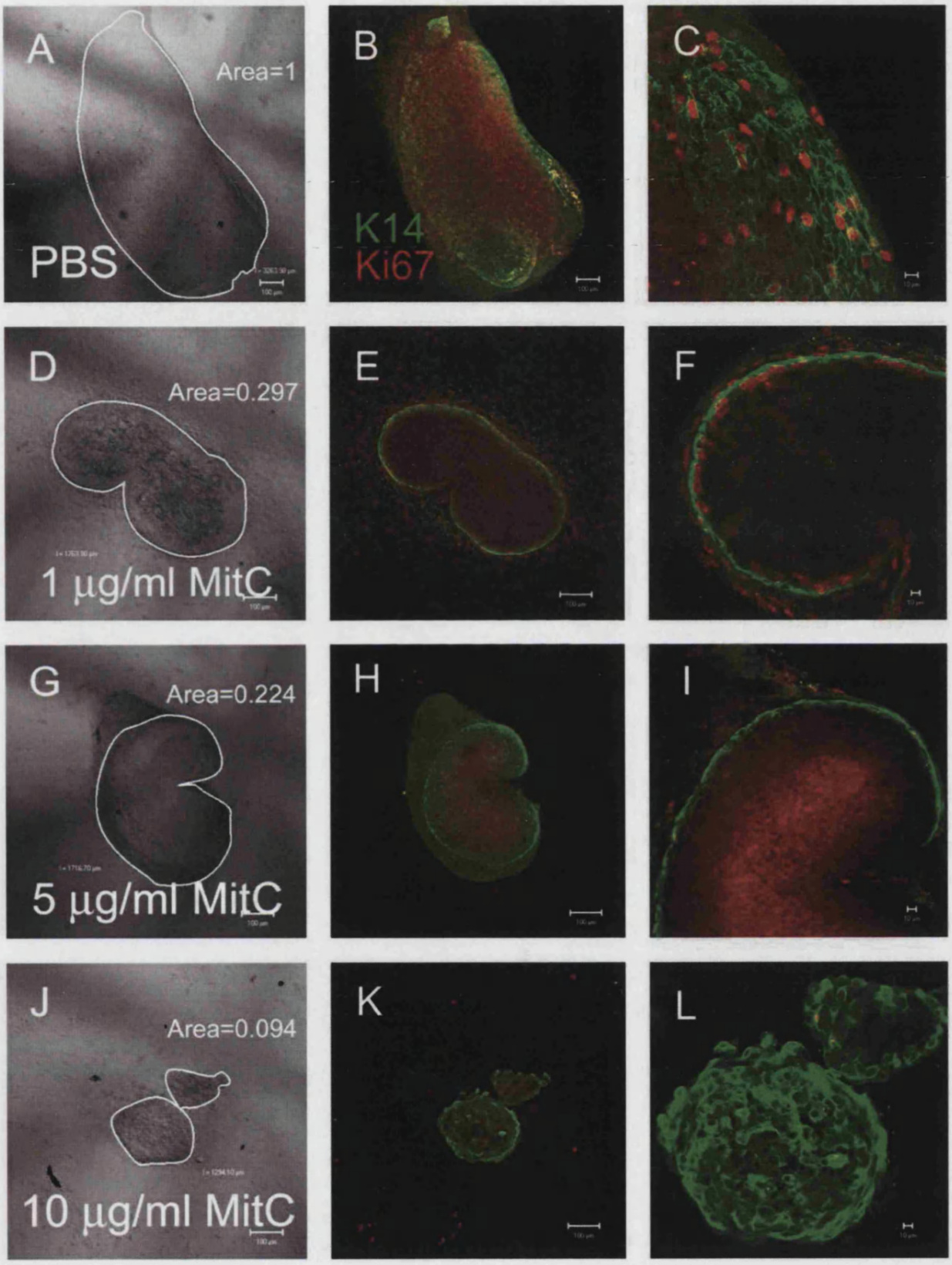

A
Area=1
PBS
B
K14
Ki67
C
D
Area=0.297
1 μg/ml MitC
E
F
G
Area=0.224
5 μg/ml MitC
H
I
J
Area=0.094
10 μg/ml MitC
K
L

Fig 4.8 Conversion of columnar to stratified squamous epithelium does not require cell proliferation.

To determine whether inhibition of proliferation had an effect on the emergence of stratified squamous cells, 0 µg/ml (PBS control)(A-C), 1 µg/ml (D-F), 5 µg/ml (G-I), 10 µg/ml (J-L) of mitomycin C were added to the oesophagus at day 4 of culture for 24 hr. After treatment, the cultures were then switched back to normal culture medium until day 9 and stained for K14 (green, FITC) and Ki67 (red, TR). PBS controls expressed many Ki67 positive cells on day 9 of culture. Many Ki67 expressing cells were found in the 1 µg/ml mitomycin C treated cultures (E, F) and a few in the 5 µg/ml treatment (H, I), but none were found in the 10 µg/ml cultures (K, L). Despite the inhibition of proliferation, K14 is still expressed in the epithelium treated with the mitomycin C. Again, (C, L) are grazing sections through the basal layer. Setting the area of the PBS control as 1, the relative area for 1 µg/ml, 5 µg/ml and 10 µg/ml mitomycin C treated cultures are 0.297, 0.224, 0.094, respectively.

4.8 Discussion

4.8.1 Summary

In this chapter, I examined the cellular origin of the stratified squamous epithelium in the developing mouse oesophagus. The organization of stratified squamous tissues such as the epidermis has been intensively studied in recent years and is quite well understood (Watt et al., 1989; Byrne et al., 1994; Jensen et al., 1999). But in the oesophagus, the embryonic origin of the stratified squamous basal layer has not been resolved. Although it has been proposed that it derives from the original columnar tissue (Thorey et al., 1993), direct evidence for this has so far been lacking.

In terms of the mechanism of the switch, I have shown that the basal layer cells do indeed derive directly from the former columnar cells. This was done by three methods, first by showing the co-expression of keratin 8 and 14 in some cells (both *in vivo* and *in vitro*); second by performing DiI labelling of columnar cells, which later show co-expression with K14, and third by showing the activation of the K14 promoter in some basal cells expressing K8. I have examined the possibility of an overgrowth of one cell type by the other by examining both the cell death and the cell division. Cell death does occur but in both populations to about the same extent. Moreover if cell death is inhibited, the change in phenotype still takes place as normal. Similarly I show that cell division is not necessary for the change, although under normal circumstances a high proportion of the cells are dividing.

4.8.2 Migration of the stratified squamous progenitor cells

An alternative possibility to the direct conversion of columnar to basal stratified cells is that the squamous epithelium cells arise from progenitors outside of the

oesophagus. The best potential site is the oral cavity that is derived from the ectoderm and endoderm, and forms stratified squamous epithelium earlier than the oesophagus (Waterman and Balian, 1980). My results show that if there is any migration from another site, the cells would have to migrate into the oesophagus before E11.5d since in the cultures the tissue is isolated around this time. It would also be a prerequisite that the progenitor cells do not express the stratified squamous markers at the time of migration, as we do not see them in either tissue from embryos or in the cultures system until after E15.5. I cannot exclude the possibility that some cells do migrate in at an early stage, or that the cells that make the transition from columnar to squamous are a separate population that migrated in before E11.5, although there is no evidence for either of these possibilities.

4.8.3 Significance of columnar cell loss

Simple columnar cells die by apoptosis if they are detached from the extracellular matrix (Gilmore et al., 2000). This is different from stratified squamous epithelia where the cells remain metabolically active for several days after they detach from the basal lamina (Fuchs and Raghavan, 2002). Generation of a single cell suspension using our method may result in a high proportion of dead cells in the K14 or K8 positive fractions and may lead to an over representation of the true death rate of the cells in culture. However, the same manipulation on these two populations of cells after four repetitions should make them still comparable. From the results comparing the dead cells treated with the caspase inhibitor and the DMSO controls (Fig 4.6) show that some of the cells are lost by apoptosis. However, the loss of columnar cells in the oesophageal epithelium is at least not dependent on the

caspase-mediated programmed cell death; this would suggest that loss of the columnar phenotype is through columnar gene regulation. I will try and identify the molecular controls for regulating columnar gene expression in the next chapter.

4.8.4 Columnar cells and apoptosis

One important function of the simple columnar keratins is that they are both targets and modulators of apoptosis (Caulin et al., 1997; Caulin et al., 2000). K18 is one of the earliest targets of caspase 3 and caspase 7 (these caspases are enzymes that cleave substrates and lead to apoptosis). After the activation of caspase 3, apoptosis will progress and reach a point of no return (Hengartner, 2000). It is likely that the fragmentation of K18 filaments by caspases may help apoptotic cells be cleared from the epithelial sheet by allowing the keratin network to be dismantled. It has also been reported that K8 and K18 are involved in attenuating and desensitising the apoptotic stimuli mediated by tumour necrosis factor-α by binding to the downstream TNFR effector-TRADD (Inada et al., 2001). K8 and K18 also modulate the Fas receptor mediated apoptosis involved in hepatotoxin induced apoptosis by acting as the substrate for c-Jun N-terminal Kinase. K8 and K18 acting as the substrates for JNK has the affect of sequestrating JNK and prevents it from phosphorylating c-jun, thereby interferes the Fas receptor signalling (He et al., 2002). This evidence shows that the simple columnar intermediate filaments have a role in both regulating and being regulated by programmed cell death.

4.8.5 Proliferative capacity of an oesophageal epithelium is within the basal layer

Previous work has attempted to locate the stem cell compartment in the epithelium of the adult human oesophagus. Seery and Watt described the papillary and interpapillary architecture of the mature human oesophageal epithelium (Seery and Watt, 2000). They demonstrated Ki67 expression in few papillary basal cells (45 out of 10,865) and even fewer in the interpapillary basal layer (9 out of 8,811). Not surprisingly, this compartmentation is not seen in the embryonic cultures, as the papillary structure is yet to develop. In the later cultures with fully stratified epithelium we find that all of the dividing cells are in the basal layer (Fig 4.7), whereas Seery and Watt found some dividing cells in the epilayers 2-3 cells above the basal layer in the mature human tissue.

4.8.6 Is it transdifferentiation?

In our case, the conversion of simple columnar to a stratified squamous tissue in the oesophagus probably does not require cell division as some K14 positive cells are Ki67 negative. However, in the current understanding of the stem cell population in the oesophagus / stratified squamous tissue, it is unlikely that all cells that have changed to stratified squamous phenotype in Fig 4.8 L are stem cells. This presumably means the non-progenitor (or at least, the partly differentiated) cell types will undergo transdifferentiation into the stratified squamous basal layer phenotype. Therefore from the results I have gathered, that cell division and cell death are not essential for our case of the conversion to occur, we would rather think this is more like a true transdifferentiation event – which is from a relatively differentiated phenotype (not the progenitor / stem cell population) directly changing to another differentiated phenotype.

4.8.7 Conclusions and the way forward

From the results of the current study, we may analyze the strength of each experiment to support the following two hypotheses: (a) whether the oesophageal epithelia developed is by a direct cell type change from columnar to stratified squamous or (b) are there two types of progenitor cells co-existed in the embryonic epithelium.

Table 4.1 Experimental evidence to support the two hypotheses of the origin of the stratified squamous epithelial cells.

Experiments	direct transdifferentiation	2 progenitor cells
Co-incidental staining	+++	+ (there might still be adult K14 cells not originally from the co-stained cells)
K14-GFP electroporation	++++	+ (there might still be adult K14 cells not originally from the co-stained cells)
Apoptosis inhibition study	++++	+ (cannot completely rule out the possibility of other outcome of the columnar cells fate, e.g. they might migrate out)
Growth inhibition study	++++	- (ruled out the hypothesis of cell replacement by another source of stratified squamous stem cell)
DNA methylation result (Fig 5.3 F and H)	++++ (by inhibiting DNA methylation which causes the natural loss of K8 , we	-

	see large scale K14 and K8 co-expression at the basal layer)	

From the results in the present chapter, I have shown using an oesophageal culture model that of the columnar cells convert to a stratified squamous phenotype probably through direct transdifferentiation. The next question in mind is to find out what are the molecular mechanisms underlying the process?

Chapter 5 Signalling and the conversion of oesophageal columnar to stratified squamous epithelium

5.1 Introduction

In the earlier chapters, I described the phenotypic changes from simple columnar to stratified squamous in the developing oesophageal epithelium. I demonstrated that the full differentiation program of a stratified squamous epithelium becomes established and showed that the stratified squamous epithelium comes directly from the simple columnar cells rather than via outgrowth of another cell population. Furthermore, inhibition of cell death or cell proliferation does not affect the transition from a columnar to stratified squamous phenotype.

In this chapter, I will focus on the following questions:

(a) Is the mesenchyme required for the transition?

(b) Do Wnt and Shh signalling regulate this process?

(c) Is there epigenetic regulation of columnar genes?

The aim of the experiments described in the present chapter is to determine the possible signalling mechanisms that control down-regulation of columnar genes and the up-regulation of stratified squamous genes.

5.2 Role of mesenchyme and Wnt / Shh signalling in the conversion

5.2.1 Overview

5.2.1.1 Epithelial-mesenchymal interaction

It is generally thought that the dermal region is important for instructing the ectodermal layers immediately above to gain the stratified squamous tissue identity (Byrne et al., 1994). For example, at E9.5, dermomyotome derived from the somite region provides signals to the ectodermal cells lying just above it to express K5 (Byrne et al., 1994). I wanted to test the idea that the mesenchymal region surrounding the oesophagus epithelium affects the differentiation of the stratified squamous epithelium. It has recently been found that the thickness of mesenchymal layers in embryonic liver cultures is reduced when cultured with Keratinocyte-serum free medium (KSFM) (Wan-Chun Li, personal communication). I tried culturing embryonic oesophagi in KSFM to see if I could reduce the mesenchymal layers and therefore the columnar to stratified squamous transition.

5.2.1.2 Morphogen signalling

To further address which signals might be emanating from the mesenchyme to the epithelia, and the effects they might have on the formation of the stratified squamous epithelium, I tried inhibitors and activators of two important signalling pathways - the Wnt pathway and the Shh pathway, (these were introduced in sections 1.2.3 and 1.2.4).

5.2.2 Results

5.2.2.1 Keratinocyte Serum-Free Medium (KSFM) and mesenchymal layers

The simplest approach to understanding whether the mesenchyme surrounding the epithelium controls the switch from simple columnar to stratified squamous epithelium is to mechanically remove the mesenchyme at the outer layers of the oesophageal epithelium. However, mechanically removing the mesenchymal layers at the E11.5 stage proved very difficult without damaging the epithelium. This is partly because there is only 1 layer of epithelial cells and the mesenchymal and pre-muscular layers are very thin. An alternative approach is to use a defined medium to suppress mesenchymal growth. The culture medium keratinocyte serum free medium (KSFM), when supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) (here named as KSFM+EGF+BPE), is suitable for keratinocyte (epidermal epithelium) outgrowth but does not support growth of mesenchymal cells (dermal cells) and is usually used for long term primary keratinocyte culture (Ura et al., 2004). Culture of E11.5d oesophagus for 7 days with KSFM plus 25 ng/ml recombinant human EGF and 25 µg/ml BPE (see section 2.2), reduced the thickness of the mesenchymal layers (Fig 5.1 A-F). Whether this was the result of loss of specific mesenchymal layer(s), or the result of specific mesenchymal cell types migrating out of the culture, or by a change to a smaller cell size in these layers, needs to be further investigated. Mesenchyme cells were stained with smooth muscle actin antibody (SMA, shown here in red, Fig 5.1 B-F). Morphologically, the treated oesophageal cultures appeared longer in length than the untreated cultures (Fig 5.1 A, G, I). This was probably due to less mechanical constraint of the mesenchymal and muscular layers on the outgrowth of the whole epithelial

A
E11.5d7
KSFM 7d
B
E-Cad
SMA
C
D
E
F
G
p63
K10
E11.5d7
KSFM 7d
H
I
E11.5d7
KSFM 7d
J
K14
K8
K
L

Fig 5.1 Effect of KSFM on oesophageal mesenchymal layers and epithelial differentiation.

Oesophagi were cultured in KSFM supplemented with EGF and bovine pituitary gland extract for 7 days. Morphologically, the oesophagus cultured in KSFM elongated and did not contain many mesenchymal layers (A, I). Dual immunostaining of E-cadherin (FITC, green) and SMA (TRITC, red) (B-F) were performed to demonstrate the relative location and morphology of the oesophageal epithelium and mesenchymal layers on different levels of the oesophagus. p63 (FITC, green) and K10 (TRITC, red) were co-stained (G, H) to show the basal layer and suprabasal differentiation of the stratified squamous epithelium. K14 (FITC, green) and K8 (TRITC, red) were stained (J-L) to show the formation of the basal layer of the stratified squamous epithelium and loss of the columnar markers after 7 days of culture with KSFM.

cells.

To examine whether reduced thickness of mesenchymal layers influenced the switch from K8 to K14 expression at the basal layers, and whether a further differentiation process of a stratified squamous epithelium is affected, I stained p63 and K10 (Fig 5.1 G, H) or K8 and K14 (Fig 5.1 I-L) in control and KSFM+EGF+BPE treated oesophageal cultures. p63 expression remains the same as control (compare Fig 5.1 H with Fig 6.5 P), while the other markers such as K14 and K10 were expressed slightly earlier than normal control (for example, compare Fig 5.6 J-L with Fig 3.6 D for K14 and K8 expression, and compare Fig 5.6 H with Fig 3.7 D, E for K10 expression). No spatial discrepancy is found between normal serum-containing medium and in KSFM culture medium. At day 7 of KSFM culture, K14 is expressed in all basal cells (Fig 5.1 K, L), K8 cells disappeared at the basal layer, and K10 cells expressed at suprabasal layers (Fig 5.1 H). Remarkably, almost all K8 positive cells at the suprabasal layers have disappeared as early as day 7 of KSFM culture (Fig 5.1 K, L), K8 usually disappears after 15 day culture under normal serum-containing medium (Fig 3.6 G, H), suggesting the reduction in mesenchyme thickness has changed the microenvironment and accelerates differentiation of the epithelium.

In conclusion, the mesenchymal thickness of the oesophageal culture is reduced after 7 days in KSFM and the formation of a stratified squamous is accelerated. The question then, is what signals from the mesenchyme are important for oesophagus epithelial development?

5.2.2.2 Wnt signalling in the developing oesophagus

I tested the effects of two important signalling pathways, the Wnt and Shh

pathways, in the epithelial development of the oesophagus.

To investigate the function of Wnt signalling in the oesophageal epithelium, I first examined the expression of E-cadherin and β-catenin both *in vivo* (Fig 5.2 A, D) and *in vitro* (Fig 5.2 B, C, E-G). I compared E-cadherin (Fig 5.2 A) and β-catenin (Fig 5.2 D) staining in adult mouse oesophagus. In the adult mouse oesophagus, E-cadherin localised to the epithelial membrane, while β-catenin was present in the epithelial and the muscle region but not clearly expressed in the submucosal layers immediately below the epithelium. The staining also showed the same distribution of E-cadherin in the epithelial region, and β-catenin in both the epithelial and mesenchymal region of the E11.5 oesophageal 7d culture (Fig 5.2 B,C vs Fig 5.2 E,F). Immunostaining in the oesophagus showed that β-catenin localises mostly with the structural E-cadherin protein near the cell surface. However, β-catenin could not be found in the nucleus even under extensive examination at higher magnification.

Lithium is known to inhibit GSK3β (Klein and Melton, 1996; Doble and Woodgett, 2003). GSK3β is the main kinase in the degradation complex to phosphorylate β-catenin and ensure its degradation. Addition of LiCl to the oesophageal culture was performed to investigate the involvement of the canonical Wnt pathway in the conversion of oesophageal epithelium from simple columnar to stratified squamous (see section 2.2). To my surprise, I still did not find any evidence of nuclear β-catenin in the treated culture (Fig 5.2 G), even when I looked at different layers of the culture using z-stack imaging on the confocal microscope. This may be because the antibody is not sensitive enough to detect the nuclear signal in the organ culture system. The normal distribution of the basal K14 cells and suprabasal cells (shown as K8 positive

cells) in the oesophagus culture is affected by 6 days of treatment with 20 mM LiCl (Fig 5.2 I) compared to control treatment of 20 mM of NaCl (Fig 5.2 H). Under these conditions, K14 no longer labels the entire basal layer but was found expressed sporadically in basal cells and is reminiscent of disorganised foci of a glandular structure (e.g. salivary gland). In contrast, K8 expression and distribution in the LiCl-treated cultures was no different compared to the 20 mM NaCl culture (Fig 5.2 H). These results suggest canonical Wnt signalling might be controlling a normal stratified squamous structure in the oesophagus.

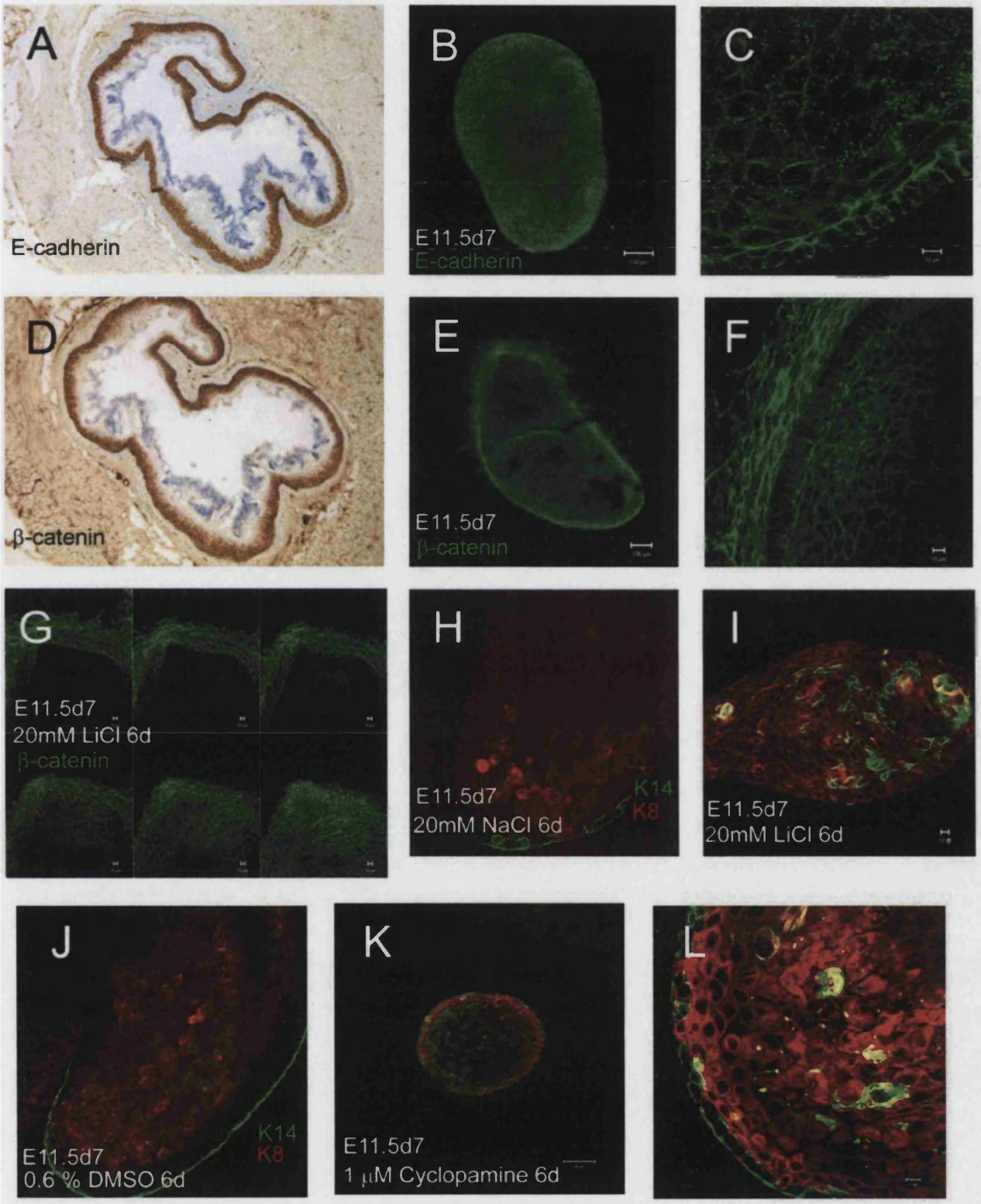

A
E-cadherin
B
E11.5d7
E-cadherin
C
D
β-catenin
E
E11.5d7
β-catenin
F
G
E11.5d7
20mM LiCl 6d
β-catenin
H
E11.5d7
20mM NaCl 6d
K14
K8
I
E11.5d7
20mM LiCl 6d
J
E11.5d7
0.6 % DMSO 6d
K14
K8
K
E11.5d7
1 μM Cyclopamine 6d
L

Fig 5.2 Activation of canonical Wnt signalling and inhibition of Shh signalling in E11.5 cultured oesophagus.

Immunostaining of E-cadherin (A-C) and β-catenin (D-F) on adult mouse oesophagus (Fig 5.2 A, D) or 7 day cultures of E11.5 oesophagi (B, C, E-G). 20 mM LiCl was added for 6 days to the 7 day E11.5 oesophageal culture, and then stained for β-catenin (G, FITC, green), or K8 (TRITC, red) and K14 (FITC, green) (I). 20 mM NaCl was added 6 days to the 7 day E11.5 oesophageal culture as control and stained for K8 and K14 (H). Note the E-cadherin only stains the membrane of the epithelial cells, while β-catenin is expressed in the mesenchymal as well as in the epithelium, but is not detected in the nucleus. The normal distribution of the basal K14 and the suprabasal K8 in the 7 day cultured oesophagus is disorganised. 0.6% DMSO (J) or 1 μM cyclopamine (K, L) was added for 6 days to the 7 day E11.5 oesophagus culture. K8 (TRITC, red) and K14 (FITC, green) were stained. The transition of the oesophageal epithelium from simple columnar to stratified squamous was not affected.

5.2.2.3 Shh signalling in the developing oesophagus

As previously mentioned (section 1.2.4), Shh is important in the bifurcation of the oesophagus and the trachea and lung. To investigate whether Shh still has a function after the separation of the oesophagus and trachea, and to determine whether it is the signal that induces the switch from simple columnar to stratified squamous epithelium, I added the Shh inhibitor-cyclopamine to E11.5 oesophageal cultures for 6 days. Cyclopamine is a steroidal alkaloid that blocks the hedgehog pathway by directly binding to Smoothened (Chen et al., 2002). Compared to the DMSO control, inhibiting the hedgehog pathway with cyclopamine did not affect the expression of stratified squamous markers and the relative structure of the oesophageal culture seems unchanged (Fig 5.2 J cf. K, L), the basal marker of the stratified squamous - K14 is expressed at the correct position in the oesophagus culture.

5.2.3 Discussion

5.2.3.1 The role of mesenchymal cells in the differentiation of the stratified squamous epithelium

In the current chapter, I tried to establish the influence of the mesenchymal cells on the development of the oesopageal epithelium. By reducing the mesenchymal layers of the oesophagus cultured in a non-serum medium, there is an acceleration of the differentiation rate in the oesophagus culture. By adding LiCl to perturb the Wnt signalling important in both the epithelium and mesenchyme of the gut, I find phenotypic changes of the epithelial morphology. Both lines of evidence demonstrate that the mesenchyme play an important role in the maturation of the oesophagus epithelium.

From the results shown in Fig 5.1, it is possible there are inhibitory signal(s) emanating from the mesenchyme that delay the differentiation of the epithelium. After 7 days of culture in KSFM, there are fewer (or smaller) mesenchymal cells and this is accompanied by premature expression of K10 and early loss of K8 (Fig 3.6 D, H and Fig 3.7 D-F). These results demonstrate that there is premature differentiation of the oesophageal stratified squamous epithelium when the mesenchymal thickness is reduced. One possible explanation is that some of the components of the KSFM medium and/or the supplements (i.e. EGF and pituitary extract) directly stimulate and accelerate the epithelial differentiation. This could be further examined by adding EGF or the pituitary extract into the normal culturing medium - BME with 20% of fetal calf serum or try the KSFM without supplement and examine the differentiation markers during different time points. However, I did not proceed further with this investigation as we do not have the composition of KSFM medium and could not fully test the hypothesis.

5.2.3.2 LiCl effect

Addition of LiCl seems to have an effect on cellular composition in the oesophageal bud culture. LiCl is an inhibitor of GSK3 (Klein and Melton, 1996; Doble and Woodgett, 2003), and probably acts through competition of Mg^{2+} ions that are normally required for the enzyme. GSK3 is involved in many different signalling pathways, such as the Wnt / β-catenin and the hedgehog pathway. It has many substrates and is known to phosphorylate β-catenin, Axin and APC in degrading the cytoplasmic β-catenin in the Wnt signalling pathway. GSK3 in concert with casein kinase 1 (CK 1) and protein kinase A (PKA), phosphorylates Ci leading to cleaving Ci into its inactive form.

LiCl is also known to inhibit the function of inositol monophosphatase and inositol polyphosphatase, thereby leading to a decreased inositol-1,4,5-trisphosphate response (Berridge et al., 1989). This inhibition prevents both recycling of inositol from inositol phosphates and *de novo* inositol synthesis, and can be overcome by extracellular inositol. While production of inositol and function of GSK3 are both inhibited by LiCl at the concentration of milli-molar range, I did not examine further on the production of inositol-1,4,5-triphosphate. To address how the cellular composition is altered by the addition of LiCl, further work should be done to first rule out the involvement of the inositols, then address which signalling pathway(s) (such as Wnt or Hh, or probably other signalling pathways) that involves GSK3β is the main cause of this change. However, from the preliminary results of blocking the Hh pathway by cyclopamine, inhibiting Shh does not seem to affect the transition and cellular composition during embryonic oesophagus development. Without the evidence for nuclear Gli staining (negative data, not shown), the

conclusion that the cyclopamine was actually functional remains to be validated.

5.3 Mechanism for down regulation of the columnar gene: Role of DNA methylation

5.3.1 Introduction

5.3.1.1 Epigenetics

Epigenetics, in contrast to genetics, is defined as meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself. DNA methylation, RNA-associated silencing and histone modification, are used to initiate and sustain epigenetic silencing (Egger et al., 2004). The role of epigenetic influences in embryonic development and organogenesis has been under intensive investigation (Li, 2002). Epigenetic mechanisms regulate many stages during development including correct gene expression in the embryonic stem cells, extra-embryonic tissue, pre-implanting and gastrulating embryos and during spermatogenesis. DNA methylation is also found to be responsible for the maintenance of genomic imprinting, and other aspects of development including gut organogenesis. Inhibitors of DNA methylation such as 5-aza-cytidine and 5-aza-deoxycytidine bind to DNA methyltransferases (Dnmt) and inhibit their function. Inhibitors of histone deacetylase (HDAC) are such as phenylbutyric acid. These are powerful chemicals affecting normal epigenetic process and are now being explored for clinical applications to suppress abnormal gene repression (Egger et al., 2004). Knockout mice for genes required for *de novo* methylation of DNA (*Dnmt3a, Dnmt3b*) are either embryonic lethal or the embryo dies soon after birth (Okano et al., 1999). Another DNA methyltransferase, Dnmt1, is responsible for the maintenance of the methylated status of the DNA (Lei et al., 1996), the homozygous knockout of *Dnmt1* causes developmental arrest and the embryos die at around E8.5. There is also evidence showing the Dnmts

co-operate in the maintenance of DNA methylation (Liang et al., 2002).

5.3.1.2 DNA methylation and changes in cell fate

Misexpression of genes could result after treatment of the DNA methylation inhibitor-5 -Aza-2 deoxycytidine (5-Aza2C). This would imply undoing the inhibition of gene expression by DNA methylation will cause unexpected genes to be expressed. This might, on occasion, cause a metaplasia. For example, adding 5-AzaC to fibroblast cell lines such as C3H 10T1/2 or NIH3T3 causes them to differentiate into a variety of cells including skeletal muscle cells, adipocytes or chondrocytes (Taylor and Jones, 1979; Konieczny and Emerson, 1984; Boukamp, 1995). There are cases when a teratocarcinoma-derived fibroblast expressed K14, and sometimes EndoA (also known as Keratin 8, TROMA-I), or even markers of corneocytes after the addition of 5-AzaC (Darmon et al., 1984), presumably as a consequence of a mesenchymal-epithelium conversion induced by 5-azacytidine. This is probably caused by inhibiting the effect of DNA methylation and re-activating specific epithelial differentiation genes (or their inducers) in fibroblasts (Semat et al., 1986).

The first intron of the *K18* gene is found to be one of the regulatory regions that transcription factors bind to and exerts its activating or repressive activities (Neznanov and Oshima, 1993). An unmethylated CpG island was found at the first exon where other heavily methylated CpG surrounds and there is a binding site for the transcription factor Ets at the 1st intron where the boundary lies. The *Ets* element was found differentially methylated in tissues that normally do not express K18 such as spleen. Transgenic mouse with 2 bp mutation at the Ets binding site enhances the mis-expression of K18 in spleen

and heart probably by disrupting the methylated sequence (Umezawa et al., 1997).

Since the mechanism for loss of columnar cells in the oesophagus does not seem to be attributable to cell death (Fig 4.6), it presumably requires a repression of columnar gene activity. I tested the hypothesis that *de novo* DNA methylation is the major cause for the columnar genes to be silenced in both the basal layer and the suprabasal layers during the transition process by treating the oesophageal culture 5-Aza2C during the switch.

5.3.2 Results

5.3.2.1 Basal and suprabasal K8 gene silencing

I began by asking if inhibition of DNA methylation altered the loss of the basal K8 expression in E11.5d oesophagus cultures (Fig 5.3 E-H). The basal layer of E11.5d oesophagus treated with 5-azacytidine (5-AzaC) for 6 days expressed the stratified squamous marker K14 as normal (Fig 5.3 A, B, D, E, F, H). However, compared with the loss of K8 marker at the basal layer in the normal oesophageal cultures (Fig 5.3 C), the treated oesophagus retained the basal K8 expression (Fig 5.3 G). This result was reproduced by treatment of oesophageal cultures with the more stable compound 5 -aza-2 -deoxycytidine (5-Aza-2C). 5-Aza-2C is dissolved in DMSO and I used that as control. 6 days of treatment with 5-Aza-2C results in the retention of the K8 expression at the basal layer (Fig 5.3 J), and K14 expression was not affected by inhibition of DNA methylation.

I extended the study to later stages of the culture period to see whether the suprabasal layer K8 expression is also regulated by DNA methylation. 5-Aza-2C was added on day 8 of culture and continued for 12 days, this is after the transition from K8 to K14 at the basal layer, but before the columnar K8 disappears at the suprabasal layers. Surprisingly, 5-Aza-2C had a dramatic effect on the suprabasal expression of K8. At the end of the 12 days treatment, when normally none or very few K8 cells were left at the suprabasal layers (Fig 5.12 K), I find most cells including some basal cells strongly expressed K8 (Fig 5.12 L), This result suggests DNA methylation is the mechanism for the cultured oesophagus to shut down columnar gene expression in both basal and suprabasal cells.

A
K14
K8
7d
B
C
D
E
5-AzaC
F
G
H
I
DMSO
7d
J
5-Aza-2dC
K
DMSO
20d
L
5-Aza-2dC

Fig 5.3 The *K8* gene is silenced in oesophageal cultures via *de novo* DNA methylation.

E11.5 oesophagi were cultured for 24 hrs and then treated for 6 days with 5 -aza-cytidine (5-AzaC) (E-H) or 5 -aza-2 -deoxycytidine (5-Aza-2C) (J, L). Or cultured for 12 days and treated from day 12 to 20 with 5-Aza-2C (K, L). PBS was used as control for 5-AzaC (A-D) and 1% DMSO was used as control for 5-Aza-2C (I, K). Fig 5.3 C, D and Fig 5.3 G, H are magnified from the same area shown by the boxes in Fig 5.3 B and F, respectively. Normally, K8 expression in the basal layer of oesophageal epithelium is reduced and few K8 positive cells are found. After adding DNA methylation inhibitors, the K8 cells are retained in the basal layer at day 7 and K14 expression is not inhibited (compare arrows in C-G and I-J). The DNA methylation inhibitors also prevent silencing of the suprabasal K8 genes when added to the cultures at the time after the basal layer has switched to stratified squamous tissue and cultured until day 20 (compare K to L).

5.3.3 Discussion

5.3.3.1 Conclusions

In this part, I wished to address whether the loss of columnar K8-positive cells was due to the suppression of K8 expression. Since it is known that DNA methylation is involved (at least in some cases) for the loss of K18 gene expression (see below), I tried adding the DNA methylation inhibitors to the cultures to see whether the columnar gene K8 is also silenced in the process of the oesophagus epithelial columnar to stratified squamous switch. I found that DNA methylation does control the loss of K8 but not the gain of K14 in the basal oesophageal epithelium (Fig 5.3 C cf G). It is somewhat surprising, however, that the silencing mechanism also acts on the suprabasal layers (Fig 5.3 K cf. L).

5.3.3.2 DNA methylation of columnar genes

DNA methylation had already been implicated in the regulation of the mouse *K18* gene (Darmon, 1985). A cultured myoblast cell line treated with 5-azacytidine results in the induction of *K18* gene. It would be of interest to understand how *de novo* DNA methyl-transferases (DNMT3a and 3b) were induced in the cultured oesophagus model, it is also of interest to determine whether they act directly on the enhancer/promoter region of *K8* or do they act upon some other upstream mechanism to control columnar gene silencing? This would mean searching the controlling elements (CpGs) in *K8* regulatory regions (Tamai et al., 1991) that has similar functional mode of *Ets* elements controlling K18 repression in the spleen and muscle. So far, we cannot rule out that there are still major changes in the context of nuclear factors during the switch and it may be a combined effect of genetics and epigenetics on the

repression of columnar genes. There are other transcription factors such as Ets members important in regulating *K8* and *K18* expression. These are Ets-1, Ets-2 and ESE-1 which are activators enhancing the expression of K8 (Hamazato et al., 1993; Umezawa et al., 1997), and Elk-3 which acts as an repressor for the expression of K8 (Nozaki et al., 1996). The oesophageal culture system provides a good model for study DNA methylation and its impact on animal development.

5.3.3.3 Is the columnar to stratified squamous a single process?

From our understanding of the initiation of the oesophageal stratification process, there is a down-regulation of the simple columnar genes (note that the two columnar markers K8 and K18 were shown in Fig 3.4) at the basal layer followed by up-regulation of stratified squamous genes, which leads to the cascade of events of epithelial differentiation and stratification. Whether the two actions are co-ordinately regulated is not known. However, the DNA methylation inhibition study showed that at least in the down regulation of the columnar genes *in vitro*, this process can be separated from the induction of stratified squamous genes (Fig 5.3 F, H, J). The preservation of the K8 expression in cells over the entire basal layer following inhibition of DNA methylation (inhibition of columnar gene silencing) also proves that the K14 cells are derived from K8-positive cells. These results also suggest that all K14-specific progenitor cells probably originate from cells expressing the K8 gene (Fig 5.3 G, H). Combined with the results of KSFM treatment (section 5.2.2), there is a remote suggestion that the silencing mechanism might be controlled by secreted factor(s) originating from the mesenchyme, i.e. when there are less mesenchymal cells, the conversion process accelerates and the

columnar genes are silenced earlier (Fig 5.1 J-L).

Chapter 6 Molecular basis of the conversion of columnar to stratified squamous epithelium in the developing oesophagus

6.1 Expression profile of candidate transcription factors

6.1.1 Introduction

6.1.1.1 Candidate transcription factors

In order to identify candidate transcription factors that might be responsible for the switch from columnar to stratified squamous epithelium, I examined a number of examples from the literature. Unfortunately, molecules and signalling pathways extensively examined for stratified squamous differentiation (the most common one is the epidermis) are mostly responsible for the process after initiation of a stratified squamous epithelium formation, which is further down the progression along the induction of a multi-layered stratified squamous tissue (reviewed in Fuchs and Raghavan, 2002). However, recent evidence showed there might be transcription factors responsible for the columnar to stratified squamous switch (reviewed in Fuchs and Raghavan, 2002). The candidates include *AP-2α*, *C/EBPα*, *C/EBPβ* and *p63*. I investigated the expression of transcription factors at the time when the transition begins and determined their location in layers of stratified squamous epithelium. I used a combination of RT-PCR and immunostaining to characterise the spatial and temporal expression of these transcription factors in the oesophagus. This allows us to gain more detailed information on the molecular basis of the switch from columnar to stratified squamous tissue.

6.1.1.2 AP-2

Currently, there are 5 members in the mammalian *AP-2* transcription factor

family: *AP-2α, AP-2β, AP-2γ, AP-2δ* and *AP-2ε*. AP-2 has a basic helix-turn-helix domain at its C-terminal involved in dimerization and sequence-specific DNA binding (see Fig 6.1 (A)) (Tummala et al., 2003). *AP-2* transcription factors play an important role in regulating gene expression during development and differentiation of multiple organs (Hilger-Eversheim et al., 2000). Known target genes of *AP-2* include the SV40 enhancer region (Mitchell et al., 1987), *c-myc* (Gaubatz et al., 1995), *p21 WAF/CIP* (Zeng et al., 1997), *c-kit* (Huang et al., 1998) and *HER-2/neu* (Bosher et al., 1995; Bosher et al., 1996). AP-2 is abundantly expressed in the skin and regulates the expression of many epidermal keratin genes (Leask et al., 1990; Leask et al., 1991) In *Xenopus*, a transcription factor found in nuclear extracts from embryos called *KTF-1* activates the epidermal keratin gene *XK81A1* (Snape et al., 1990). Later proven by Leask et al., *KTF-1* in *Xenopus* (or *KER-1* in humans) is actually the same *AP-2* (Leask et al., 1990; Leask et al., 1991). *AP-2α* is important in the expression of *K14* in the basal layer of stratified squamous epithelium (Leask et al., 1991), and functionally active binding sites of *AP-2* exist in many epidermal genes (Byrne et al., 1994; Luo et al., 2002).

AP-2 can act as an activator or a repressor depending on the target gene. For example, *AP-2* acts as activator for genes such as *p21 WAF/CIP* (Zeng et al., 1997), *HER2/neu* (Bosher et al., 1995; Bosher et al., 1996), *c-kit* (Huang et al., 1998) and many of the keratinocyte-specific genes, but down regulates the expression of *c-myc* (Gaubatz et al., 1995), *keratin 3* (Chen et al., 1997) and *MCAM/MUC18* (Bar-Eli, 2001).

AP-2 is probably best known as a tumour suppressor gene. In melanoma cells, *AP-2* up-regulates *p21*, *c-kit* and *E-cadherin* expression (Bar-Eli, 2001). These genes are important in controlling cell cycle progression, mediating cellular

differentiation, maintaining the epithelial integrity and inhibiting metastasis (Bar-Eli, 2001). *AP-2* also down-regulates the expression of *MCAM/MUC18*, a surface marker for melanoma, and the proto-oncogene - *c-myc*.

The repression of *c-myc* is probably because AP-2 directly inhibits the DNA binding activity of c-myc. AP-2 competes with an overlapping DNA binding site next to E box elements (the usual *c-myc* DNA binding site) (Gaubatz et al., 1995). Also, the C-terminus of AP-2 interacts with the dimerization domain of c-myc and impairs c-myc DNA binding activity. A similar mechanism was found where the *AP-2* binding site is next to and overlaps with other transcription factor binding sites, such as neighbouring *SP1* elements in the regulation of the corneal keratinocyte specific gene-*keratin 3* (Chen et al., 1997).

AP-2 can also be activated by oncogenes, as well as activating oncogenes. AP-2 is found in breast tumour cells to activate the *c-erbB-2, HER2/neu* receptor, which is seen as an oncogene and is related to breast cancer. Although *AP-2* RNA is induced by the oncogene *n-ras* in teratocarcinoma cells (Kannan et al., 1994), however the transactivation activity of *AP-2* is inhibited (Kannan et al., 1994; Kannan and Tainsky, 1999). In short, the *AP-2* family is closely linked to activities of controlling cell cycle and regulation of apoptosis (Hilger-Eversheim et al., 2000).

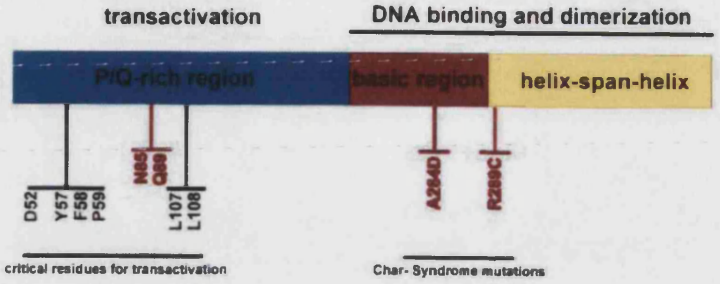
It is not entirely understood whether different *AP-2* isoforms are redundant or have specific functions in tissues where their expression overlaps. From the study of knockout mice of individual isoforms of *AP-2*, it was found that *AP-2α* knockout mice showed defects in craniofacial skeletal formation, cranial neural tube closure, failure in body wall closure, defective formation in organs such as lens and die perinatally (Schorle et al., 1996; Zhang et al., 1996). However, the *AP-2β* knockout mice were found to have rather different defects specifically in

the kidneys, and they are probably due to apoptotic cell death of the renal epithelium rather than failure of kidney cell differentiation (Moser et al., 1995; Moser et al., 1997). The *AP-2β* knockout mice die of polycystic kidney disease shortly after birth.

In stratified squamous epithelium, *AP-2* isoforms are found in different cell types. AP-2α is found predominantly in the basal layer. AP-2B, an alternative spliced inhibitory form of AP-2α is found expressed in the mesenchyme underneath the basal layer (Byrne et al., 1994). AP-2γ is expressed in all layers of the epidermis, and can activate a cystein proteinase inhibitor-cystatin A expressed in the cornified layers (Takahashi et al., 2000). Recently, AP-2ε was also found to be highly expressed in the skin, and binds to the *AP-2* binding sites of the keratin genes in keratinocytes (Tummala et al., 2003).

It is assumed that the molecules involved in regulating differentiation of the epidermis will most likely do the same job in the oesophagus. AP-2α may induce differentiation of the epidermis and induction for K5 and K14 production. *In vitro* results have shown that AP-2α is necessary but not sufficient to induce *K14* gene expression (Byrne et al., 1994). However, *AP-2α* knockout mice showed no apparent defect in the skin, and the keratin gene expression was unaffected (Talbot et al., 1999). The results suggest other members of the *AP-2* family compensate for the loss of *AP-2α*. This in turn, makes it more difficult to decipher the function of *AP-2* in the skin. AP-2α is an important factor in mediating and maintaining the expression of cytoskeleton of stratified squamous epithelium, I used AP-2α both as a positive control for testing induction of the *K14* promoter and also examined whether it will repress *K18* promoter expression in the basal layer.

(A)



AP-2 family

(B)

p63 family

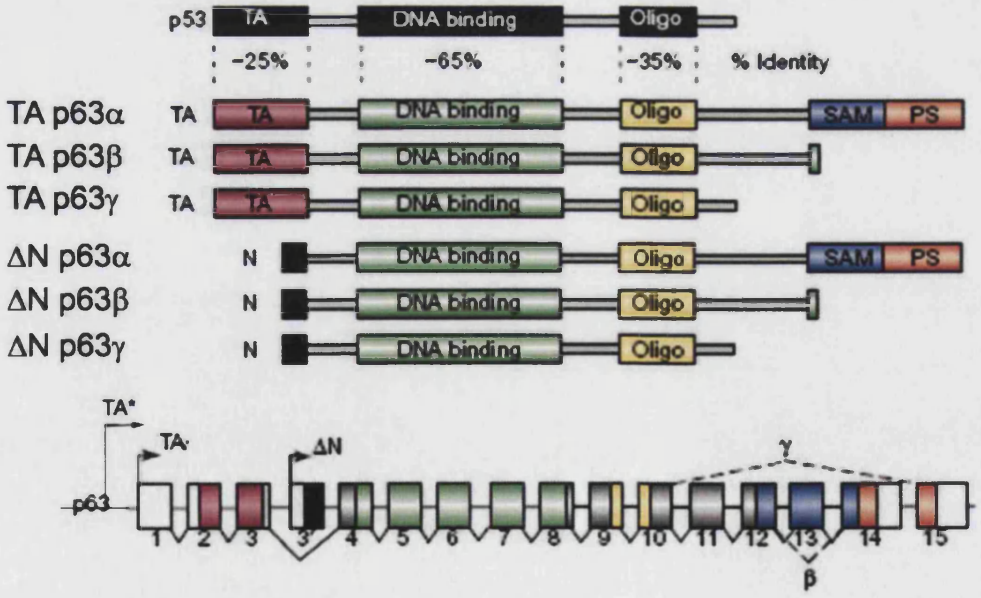


Fig 6.1 Diagrams showing the gene structure of *AP-2* and *p63* isoforms.
(A) Transcription factors of the *AP-2* family have a common transactivation domain at the N-terminus, and a basic helix-span-helix DNA binding and dimerization domain at the C-terminus. Adapted from Gene, Vol. 260, p1-12 (Hilger-Eversheim et al., 2000). (B) *p63* transcription factor has a similar transactivation domain, DNA binding domain and oligomerization domain as the transcription factor, *p53*. However, the many isoforms of *p63* includes alternative transcription start site at the N-terminus giving rise to transactivating (TA) isoforms, transactivating star (TA*) and delta-N (ΔN) isoforms; and alternative splicing to either include the sterile-alpha motif (SAM) and the post-SAM (PS) domain or just the shorter C-terminus domains give rise to α, β and γ isoforms. There are at least 6 different possible isoforms for the transcription factor *p63*. Adapted from Trends in Genetics, Vol. 18, p90-5 (Yang et al., 2000).

6.1.1.3 C/EBPs

AP-2 and members of the *CCAAT/ enhacer binding protein* (*C/EBP*) family of transcription factors co-ordinately control the expression of K1 and K10 (Maytin and Habener, 1998; Oh and Smart, 1998; Zhu et al., 1999). *C/EBP*s are members of the leucine zipper family (bZIP) of transcription factors and are important in cellular differentiation. The *C/EBP* transcription factors are expressed in hepatocytes, keratinocytes, adipocytes and haematopoietic cells (Lekstrom-Himes and Xanthopoulos, 1998). In the epidermis, for example, C/EBP is expressed at the epithelium and was proved to be inhibitory to the basal layer specific α2-integrin (a collagen receptor) (Corbi et al., 2000).

Maytin et al. (1999) found that K10 is upregulated in suprabasal layers upon loss of AP-2α. The loss of AP-2 might be due to cells receiving differentiation signals as they migrate upward (Maytin et al., 1999). Additionally, Maytin et al (1998) found that AP-2α and C/EBPβ are expressed at the basal layer of stratified squamous tissue, and C/EBPα and K10 are expressed in the suprabasal layers (Maytin and Habener, 1998). In adipocytes, AP-2 is known to repress C/EBPα, which is an important regulatory gene for adipogenesis (Jiang et al., 1998). Maytin et al. thus hypothesised that C/EBPβ controls the expression of AP-2α which in turn represses the expression of C/EBPα at the basal layer. When the cells of the basal layer move upward, C/EBPβ is no longer expressed and neither is the C/EBPα repressor-AP-2α. C/EBPα is activated and subsequently controls the expression of the differentiation marker K10. Furthermore, biochemical analysis demonstrates that C/EBP protein interacts with the *K10* promoter (Maytin and Habener, 1998). However, there is contradictory evidence on the location of different members of the C/EBP family. Smart and colleagues also found that C/EBPs regulate

expression of the early differentiation stratified squamous differentiation markers - K1 and K10 (Oh and Smart, 1998; Zhu et al., 1999). The main difference between the observations from the two groups is the location of C/EBPα and C/EBPβ. Smart et al found that C/EBPα is expressed in the basal layer and C/EBPβ is expressed in the suprabasal layers. They showed that it is the C/EBPβ that inhibits the basal cell growth and induces early differentiation (activation of the *K1* and *K10* genes). But C/EBPβ has no effect on other 'late' differentiation markers including involucrin and loricrin. A different source of *C/EBPβ* knockout mice was used by Smart's group compared to Haebner's group. Morphologically, the skin of Smart's knockout mice does show a thickened epithelia, mainly at the nucleated layers (basal, spinous, granular layers). More proliferating cells at the basal layer in the skin of *C/EBPβ* knockout mice than the wildtype suggests *C/EBPβ*'s nature in promoting epidermal cell's early differentiation and inhibit its proliferation. Based on these results, I wanted to investigate if any of the *C/EBP* transcription factors have a role in regulating expression of *K18* and *K14* genes during oesophagus development.

6.1.1.4 p63

p63 is a candidate for the master switch gene in the conversion of oesophageal columnar to stratified squamous epithelium. As a newly found member to the *p53* family, *p63* is ancestral in evolutionarily terms to the previously found *p53* (Yang et al., 2002). Structurally, like p53, p63 has an acidic N-terminal transactivating domain, a highly conserved core DNA-binding domain, and a C-terminal oligomerization domain (see Fig 6.1 (B)) (Yang et al., 1998). However, studying *p63* is complicated because there are at least 6

isoforms where there is only one single type of *p53*. The use of alternative promoters and transcriptional start sites, gives rise to an N-terminal transactivating (TAp63) and a non-transactivating (ΔNp63) isoform. In addition, alternative splicing creates three different C-terminal isoforms, designated α, β and γ (see Fig 6.1 (B)). *p53* is an essential gene for cell cycle and apoptosis control, and mainly functions as a tumour suppressor gene but is not lethal when knocked out (Hall and Lane, 1999). Because of the essential need for *p63*'s functions established in knockout studies, scientists speculate *p53* evolved later than *p63*. Further evolutionary studies have confirmed this idea (Yang and McKeon, 2000; Yang et al., 2002).

p63 is normally expressed at the basal layer of epithelia that form stratified squamous tissue (Mills et al., 1999), e.g., epidermis, oesophagus and cornea (Yang et al., 1999). p63 is also expressed during the limb bud stage especially at the apical ectodermal ridge (AER). p63 is found in the epithelium of the urothelium and prostate, in the epithelium of secretory glands such as mammary, lacrymal and salivary glands and at the branchial arch during embryonic development. The *p63* knockout mouse has no stratified squamous epithelia and the mice die soon after birth probably due to dehydration. There are no hind limbs and truncated forelimbs in the knockout mice and the teeth, hair follicles and mammary glands are missing (Yang et al., 1999; Mills et al., 1999).

There is a debate about the function of p63 in the basal layer of the stratified squamous epithelium (McKeon, 2004). Is p63 crucial for the maintenance of the stem cell population in the basal layer, or does it determine the fate of stratified squamous cells? From the two groups that performed the knockout studies in mice, the exons containing the main DNA binding domain were

either deleted or frame-shifted in the knockout mice (Yang et al., 1999; Mills et al., 1999). The results of these two groups are quite similar, with defects in limb, tooth and craniofacial formation, and apparent malformation of all stratified squamous tissue. However, there were some differences between the two models. First, terminal differentiation markers at the site of stratified squamous epithelium were detected by McKeon's group but not Bradley's group. Yang et al (1999) detected loricrin in the knockout mice and suspects that the function of *p63* is in maintaining the stem cell population at the basal stratified squamous layer (Yang et al., 1999). Mills et al (1999) did not find any differentiated stratified squamous markers in the skin and naturally concluded that *p63* is the epithelial master regulatory gene in the commitment to stratified squamous tissue (Mills et al., 1999).

The subtle phenotypic differences in the knockout mice could at least be partly explained by different functions of the *p63* isoforms (Koster et al., 2004). The TAp63 isoform is first to be expressed during embryogenesis and is required for stratified squamous cell fate commitment. Misexpression of TAp63 in some columnar cell lines such as Ptk2 (kangaroo kidney cells) or in the lung of the transgenic mice under the control of the *Surfactant Protein-C* (*SP-C)* promoter, induces the epithelia to express K14, and inhibits terminal differentiation of the skin. In contrast, ΔNp63 is expressed later than TAp63 and is responsible for maturation of the stratified squamous tissue and maintenance of the proliferative potential of mature epidermis. The possibility of different functions of the *p63* isoforms (to facilitate either the initiation or maintenance of the stratified squamous) allows compatible interpretations for the discrepancies of the two knockout models to be made.

Many people use *p63* as a stratified squamous stem cell marker (Pellegrini et

al., 2001; Moore et al., 2002; Green et al., 2003; Chen et al., 2004; McKeon, 2004). This might be due to the ability of *p63* (as a member of the *p53* family) to regulate cell cycle and apoptosis (Yang et al., 1998; Yang and McKeon, 2000; Yang et al., 2002; Flores et al., 2002). The transcription factor *p53* is generally seen as a tumour suppressor gene and can promote cell death in certain conditions. p53 is structurally similar to p63, but there is no sterile alpha motif domain (SAM) and no post SAM domain in the C-terminal of p53 (see Fig 6.1 (B)). p63 and p73 (another member of the *p53* family), can bind directly to *p53* binding sites, and act as an inhibitor of *p53* function because of either competition for binding sites or the inability to transactivate *p53* target genes (Yang et al., 1998; Yang et al., 2002; Flores et al., 2002). For example, a recent finding suggests that ΔNp63 might directly regulate a proapoptotic gene – *IGFBP-3* in stratified squamous tissue (Barbieri et al., 2005).

A description of p63 expression in the developing mouse oesophageal and tracheobronchial epithelium has been published (Daniely et al., 2004). There is also a study on the expression of p63 in human oesophageal biopsy samples from both normal oesophagus and from patients with Barrett's oesophagus (Glickman et al., 2001). Expression of keratin 14 and p63 was demonstrated in both studies, and p63 disappears once the metaplastic phenotype commenced. In normal human oesophagus epithelium, p63 is expressed mostly in the basal layer but can also be found in suprabasal cells of the oesophageal epithelium. To date, the target genes of p63 in the stratified squamous tissue are not fully understood. The most recent results show that p63 can activate a desmosomal protein - Perp at the adheren junction in the basal layer cells of stratified squamous tissue (Ihrie et al., 2005). Oddly enough, no one has determined whether p63 directly activates K14, which seems logical for a direct target of

p63. In this study I used cell lines and investigated (using luciferase reporter assay) whether p63 inhibits K18 and activates K14 expression.

6.1.2 Results

6.1.2.1 RT-PCR and immunostaining

I determined the change in expression of the transcription factors (*AP-2α*, *C/EBPα*, *C/EBPβ* and *p63*) during the conversion from columnar to stratified squamous epithelium. These changes should correlate with alteration in the time of appearance of K8 and K14. I used two approaches to address this problem. First, I examined by RT-PCR the temporal profile of the transcription factors' RNA expression during oesophagus development. Second I performed antibody immunostaining to detect the protein expression and cellular location in the tissue. RT-PCR and immunostaining of the transcription factors were carried out at least twice.

I isolated whole oesophagus (which included epithelium, mesenchymal and muscular layers) from E11.5, E13.5, E15.5, E17.5 embryos, postnatal day 1 newborn mice and from adult mice. Total RNA was extracted and reverse transcription polymerase chain reaction (RT-PCR) was used to analyse the expression of the candidate transcription factors and stratified squamous-specific markers (Fig 6.2). As indicated in chapter 2 (see section 2.7), I used 35 cycles for the PCR reaction of transcription factors and 28 cycles for the keratins and the loading control *GAPDH*.

First I determined the expression profile of *K14* and *K18*. The RT-PCR time course confirmed our results from the immunostaining of the two keratin markers *in vivo* (Fig 3.4). *K18* was expressed from E11.5 until P1, but was not present in the adult oesophagus. However, *K14* RNA is detected earlier (E13.5) compared to the protein (E15.5 to E17.5). Whether this is because the protein detection by immunostaining is less sensitive, or that translation starts about 3

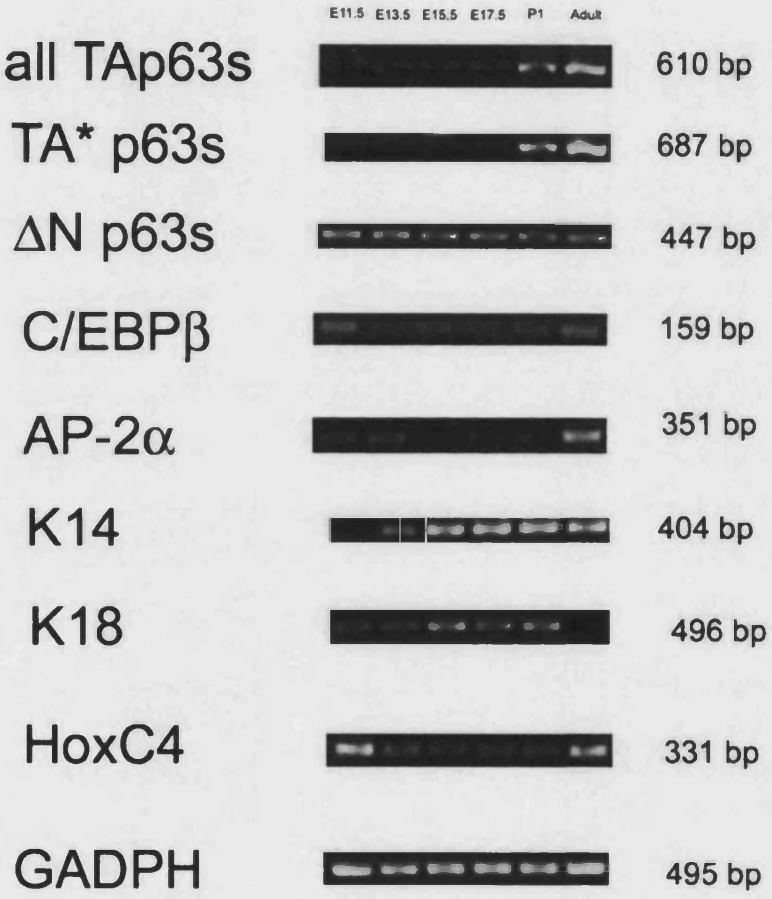

E11.5 E13.5 E15.5 E17.5 P1 Adult
all TAp63s
610 bp
TA* p63s
687 bp
ΔN p63s
447 bp
C/EBPβ
159 bp
AP-2α
351 bp
K14
404 bp
K18
496 bp
HoxC4
331 bp
GADPH
495 bp

Fig 6.2 The time course of embryonic oesophagus epithelium gene expression by Reverse Transcription - Polymerase Chain Reaction assay.

Analysis of the expression of transcription factors and columnar and stratified squamous markers during development and in adult tissues. The results shown are representative of 2 separate experiments.

days after the transcripts are produced is currently not known. The *K14* expression persists until adult stage. Next, I determined the expression of the homeobox gene - *HoxC4*, known to be expressed only in the oesophageal region of the gut (Boulet and Capecchi, 1996). *HoxC4* is expressed at all the time points I examined, showing that I indeed have isolated the oesophageal tissue (see Table 1.1).

6.1.2.2 AP-2

AP-2α, was detected by RT-PCR from E11.5 (Fig 6.2), although expression appeared to drop after E15.5, or just before the oesophagus starts to express the K14 protein (Fig 3.5 K, L). However, the strong expression of *AP2α* is found again at the adult stage both by RT-PCR (Fig 6.2) and immunostaining (Fig 6.3 L). The monoclonal antibody to AP-2α did not detect oesophageal expression at stages E11.5 to P1 (Fig 6.3 A, C, E, G, I, K and L). AP-2α is expressed in the epidermal epithelium from E11.5 (Fig 6.3 B, D, F, H and J) and in the edge of the limb bud which includes the apical epidermal ridge (AER) (E11.5 limb Fig 6.3 B). The location of AP-2α expression in both the epidermis and the adult oesophageal epithelium is the same, which is in most basal epithelial cells and some of the suprabasal layers. As found previously, there are alternative spliced forms of *AP-2* (Buettner et al., 1993; Huang et al., 1998). *AP-2B* (or called *AP-2α2* (Meier et al., 1995)) is an inhibitory form of *AP-2A* that lacks the DNA binding domain, and there might be a regulatory function of this inhibitory form to *AP-2α*. Our primers could not differentiate between the *AP-2α* and *AP-2α2*. From the results obtained by the two methods, I suspected the *AP-2α* I detected in the oesophagus by RT-PCR at the early embryonic stages is the inhibitory isoform of *AP-2α*, but this inhibitory isoform may not be

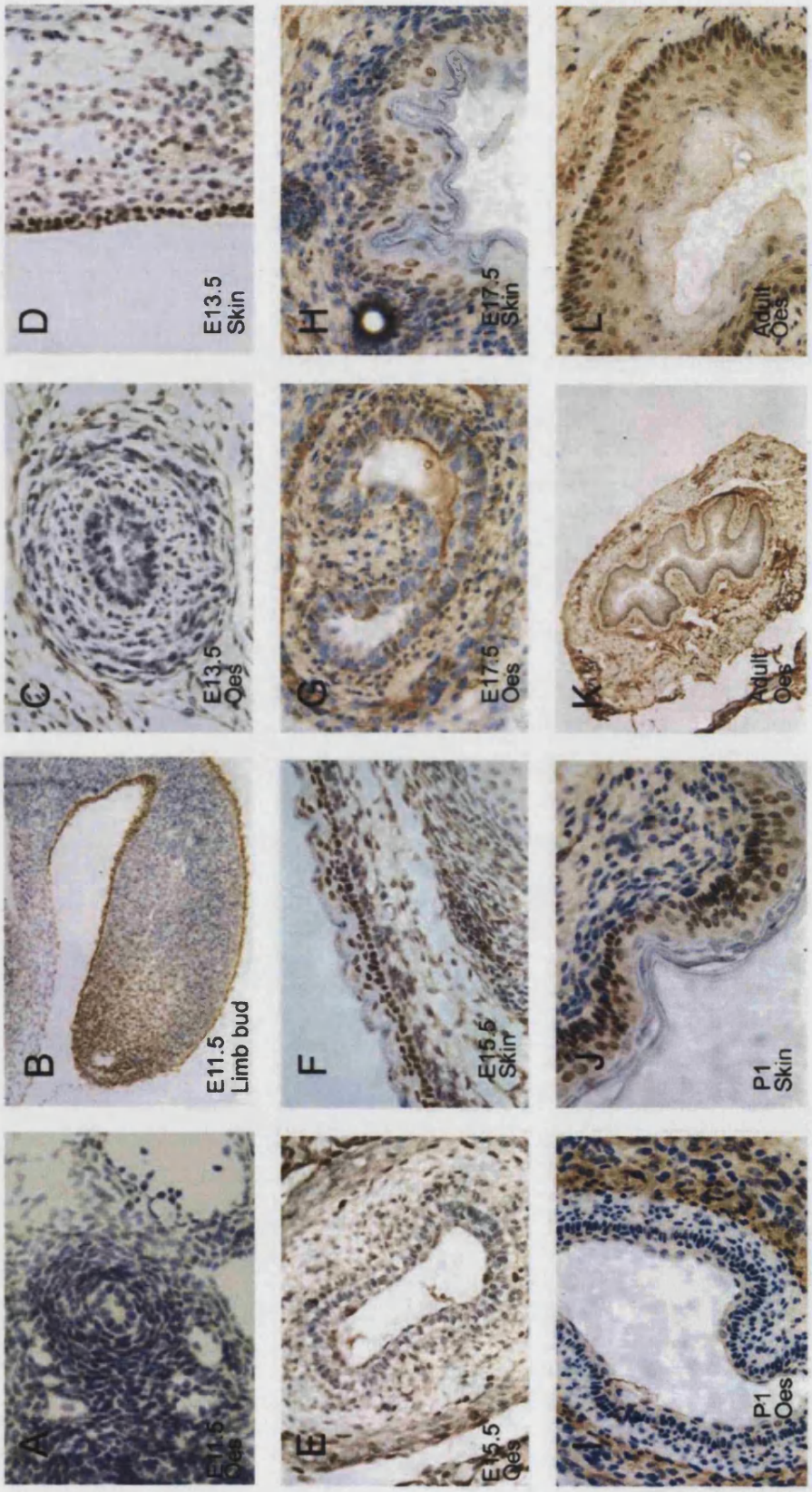

A
E15.5
Oes
B
E11.5
Limb bud
C
E13.5
Oes
D
E13.5
Skin
E
E15.5
Oes
F
E15.5
Skin
G
E17.5
Oes
H
E17.5
Skin
J
P1
Skin
K
Adult
Oes
L
Adult
Oes
P1
Oes

Fig 6.3 Expression of AP-2α in the oesophagus and skin during development. Immunostaining of AP-2α on cross sections of embryos from E11.5 to adult (2 months old) mice, either on oesophageal epithelium (A, C, E, G, I, K, L) or skin epithelium region (B, D, F, H, J). AP-2α marks many of the cells on the apical ectodermal ridge region of the forming limb bud (B). The basal layer of the skin showed strong expression of AP-2α from E11.5, but the basal epithelium of the oesophagus only showed AP-2α expression in the adult oesophagus and not before perinatal stage.

detected by antibody staining. However, this needs further confirmation and I still cannot exclude the possibility that other AP-2 isoforms could initiate K14 expression in the oesophagus.

6.1.2.3 C/EBP

The RT-PCR results show that *C/EBPβ* was expressed from E11.5 until the adult stage. However, I failed to detect *C/EBPα* in the oesophagus by RT-PCR methods (although it was detected in the adult liver, which is known to express this gene, data not shown). Immunostaining of the adult oesophagus (Fig 6.4) showed an expression pattern similar to the findings of Smart et al. in the skin (Oh and Smart, 1998; Zhu et al., 1999). C/EBPα is expressed in the cytoplasm or perinuclear region in most cells of the basal layer, and in the nucleus of some suprabasal cells (arrows in Fig 6.4 B). C/EBPβ is expressed in very few cells in the basal layer, and in more cells of the suprabasal layers (all in the nucleus) (Fig 6.4 D).

Although there are some discrepancies as to the role of C/EBPs in the regulation of keratin genes in stratified squamous, there is no doubt *C/EBP*s are important for keratin gene expression and stratified squamous differentiation. It is possible that the basal C/EBPα and the suprabasal C/EBPβ have a role controlling the simple columnar to stratified squamous conversion, perhaps in the down-regulation of *K8* and *K18* columnar genes. I will investigate this possibility using the luciferase assay (see section 6.2).

6.1.2.4 p63

Koster et al. (2004) showed that TAp63 is expressed earlier than ΔNp63 in tissues of the embryos that later become stratified squamous epithelium. Using

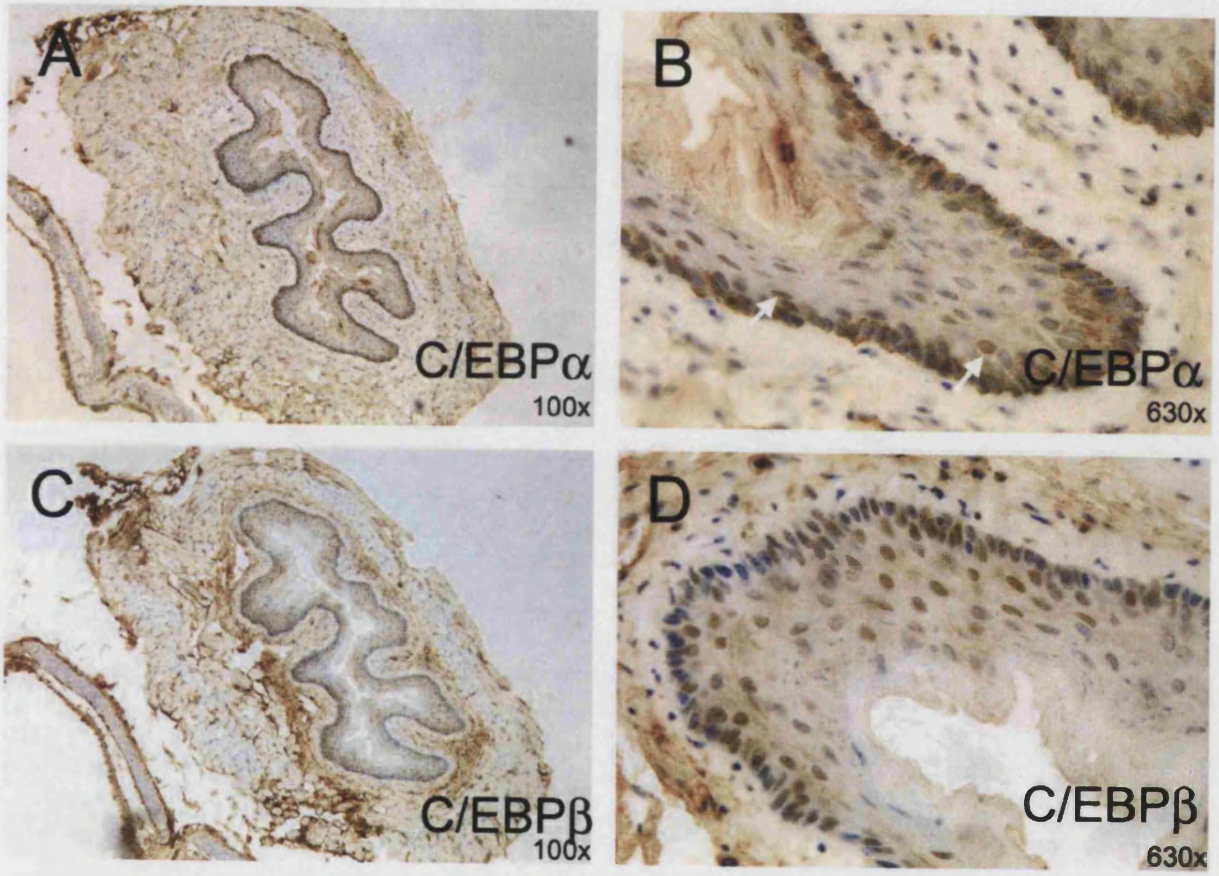

A
C/EBPα
100x
B
C/EBPα
630x
C
C/EBPβ
100x
D
C/EBPβ
630x

Fig 6.4 Expression of C/EBPα and C/EBPβ in the oesophagus epithelium.

In the adult oesophagus, C/EBPα is mainly expressed in the basal layer, and to a lesser extent in the suprabasal layers (A, B). The basal cells showed perinuclear expression of C/EBPα, while some of the suprabasal cells showed expression of C/EBPα in the nucleus (arrows in B). C/EBPβ is found expressed in the nucleus of cells at the suprabasal layers of oesophageal stratified squamous (Fig 5.4 C, D).

RT-PCR, the transactivating isoforms were shown to be expressed from E7.5, and the ΔN isoforms, expressed from E9.5 are the dominant form around birth (Koster et al., 2004). It is worth noting that there is another additional 39-amino acid stretch at the N-terminal of p63 (Yang et al., 1998), named TA* isoform, the function of which is unknown. I specifically designed PCR primers to detect the TA* isoform (but not the TA or ΔN isoforms), while another set of primers detected all the TA isoforms (including the TA* isoform). A third set of primers were designed to specifically detect only the ΔN isoforms since there is a region present in the ΔNp63 N-terminal that is not present in any of the other TA isoforms (because of alternative selection and splicing of the 3rd exon). Using RT-PCR, the results for the different *p63* isoforms were rather surprising. I find no significant quantity of any of the *p63* TA isoforms (the design of the primers will not distinguish between α, β and γ isoform) until after P1 (Fig 6.2). However, the TA* specific isoform was detected at the same time points as the general TAp63 isoforms were detected (P1 and the adult stage oesophagus). On the other hand, the ΔN isoforms are strongly expressed at all the time points examined. This suggests that in the oesophagus, the ΔN isoforms are already dominantly expressed from the start of the observation period, i.e. E11.5. In contrast, the TA isoforms of *p63* are hardly detectable before birth, and the major *p63* isoform with the transactivation domain is the TA* isoform.

I next examined the spatial location of p63 by immunohistochemistry and immunofluorescence detection methods (Fig 6.5). The antibody I used detects all the p63 isoforms. Immunostaining was performed on sections of mouse E11.5, E13.5, E15.5, E17.5, P1 and adult oesophagus. p63 was strongly expressed at the basal layer and in a few cells of the suprabasal epithelium. While the expression of *p63* by RT-PCR and immunohistochemistry shows p63

protein expressed at the basal layer from E11.5, there might be changes of different p63 isoforms expressed. So far, I do not have a method to distinguish between α, β and γ isoforms. We will need a better understanding of the interaction of the different p63 isoforms and the whole p63 story may be more complicated than it seems by just using the pan-p63 antibody.

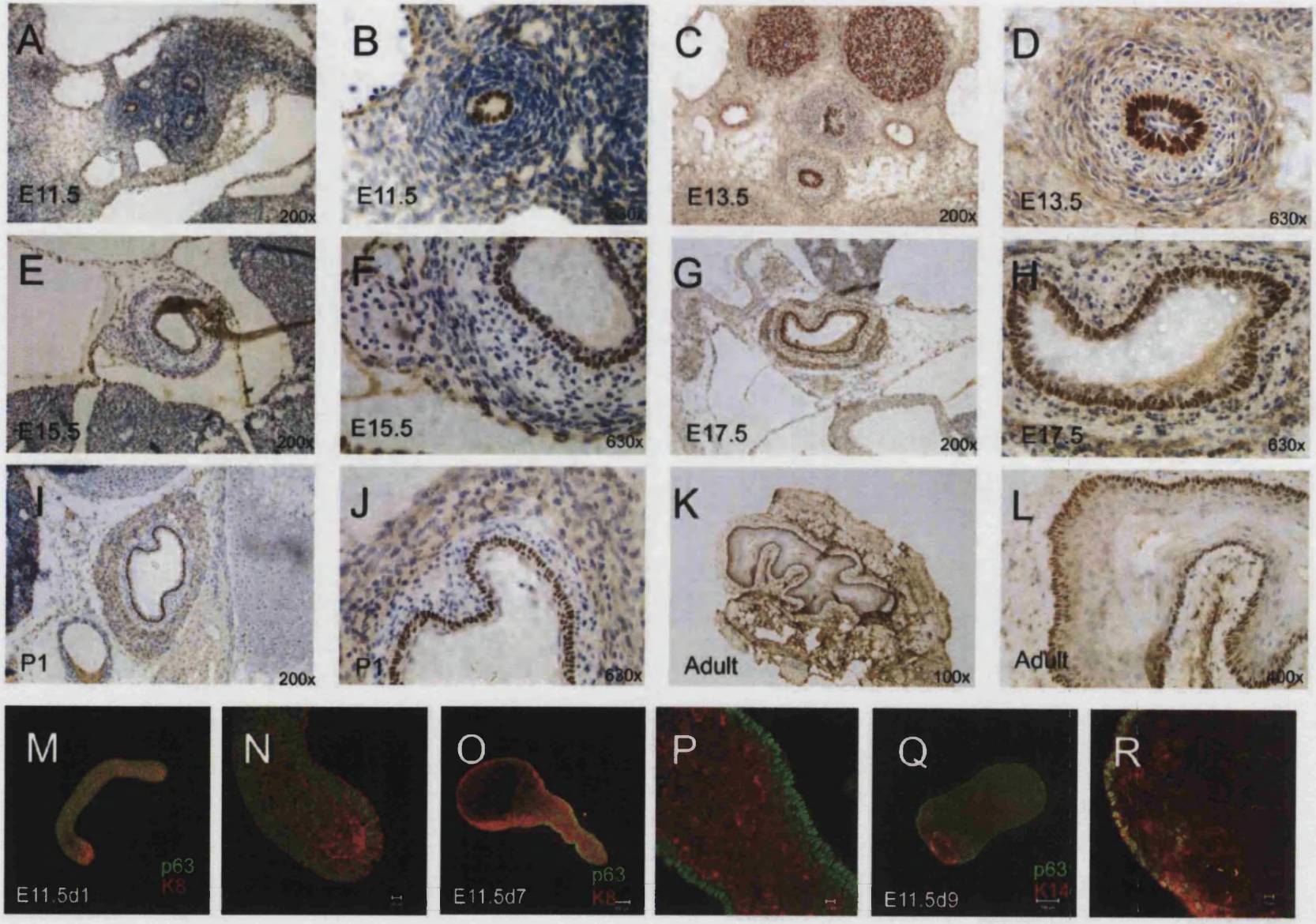

A
E11.5
200x
B
E11.5
C
E13.5
200x
D
E13.5
630x
E
E15.5
200x
F
E15.5
630x
G
E17.5
200x
H
E17.5
630x
I
P1
200x
J
P1
630x
K
Adult
100x
L
Adult
400x
M
p63
K8
E11.5d1
N
O
p63
K8
E11.5d7
P
Q
p63
K14
E11.5d9
R

Fig 6.5 Expression of p63 in the oesophagus *in vivo* and *in vitro*.

The p63 antibody stains the nucleus of nearly all basal layer cells of the oesophageal epithelium from E11.5 to the adult stage (A-L). Note nuclear p63 is also stained in the trachea and bronchial regions at E13.5 (C). Embryonic oesophagi isolated from E11.5 mouse were cultured in vitro for 1 day (M, N), 7 days (O, P) and 9 days (Q, R). K8 (red) and p63 (green) were co-stained at 1 day (M, N) and 7 days culture (O, P) to show the co-expression of both markers at the beginning of the culture, but do not co-exist in the same cells after the stratified squamous programme has started. K14 and p63 were co-stained at 9 days culture (Q, R) showing co-expressing of both markers at the basal layer of the stratified squamous oesophageal epithelium.

6.1.3 Discussion

6.1.3.1 Conclusions

I used two methods: RT-PCR and immunohistochemistry to determine the time course of expression of the candidate transcription factors: *AP-2α*, *C/EBPα*, *C/EBPβ* and *p63*s. Information on the spatial and temporal expression profile of these genes was obtained. However, some questions are raised after comparing the results of the two methods. Based on immunostaining, I showed that all the transcription factors are expressed in either the basal layer (AP-2α, C/EBPα and p63), or the suprabasal layers (C/EBPβ). However, studying the time course of expression of different isoforms of each transcription factors is much more complicated.

The results from RT-PCR and immunostaining studies suggest the AP-2α isoform is not expressed in the oesophagus until postnatal stages. This suggests the mRNA signal detected at earlier stages may be the inhibitory isoform of *AP-2α* - *AP-2α2* (*AP-2B*). I could not detect *C/EBPα* by RT-PCR, but C/EBPα is found in the basal layer of the adult oesophagus by immunostaining. The RT-PCR results for the time course of *C/EBPβ* expression showed the transcription factor is present from E11.5 to the adult stage. I cannot rule out the possibility that the *C/EBP* signals from RT-PCR were amplified from the vascular smooth muscle and the stromal layers of the oesophagus during sample collection (Kousteni et al., 1998; Sekine et al., 2002). Here follows a brief discussion of implications we can make of for each transcription factor.

6.1.3.2 AP-2α

Although AP-2α could not be detected by immunostaining, it was present based on RT-PCR results. There is evidence of the inhibitory AP-2α2 found in

the dermis of the skin at E13.5 (Byrne et al., 1994). If the form of *AP-2α* detected by RT-PCR is the inhibitory form - *AP-2α2*, it is probable that other *AP-2* isoforms are involved in the formation of the oesophageal epithelium. Recently, a new *AP-2* isoform (called *AP-2ε*) is expressed in all layers of the epidermis (Tummala et al., 2003). Whether *AP-2ε* is involved in the expression of the stratified squamous specific keratin genes remains to be determined.

6.1.3.3 C/EBP

From the results of C/EBP expression profile in my study, I found C/EBPα expressed at the basal layer and C/EBPβ at the suprabasal layers. This is similar to the results by Smart's group but not Haebner's group (Oh and Smart, 1998; Maytin and Habener, 1998; Zhu et al., 1999; Maytin et al., 1999). In this case, the role of the basal C/EBPα could be either activating or suppressing the basal proteins of a stratified squamous tissue. Surprisingly, I find that there is perinuclear or cytoplasmic staining of C/EBPα in cells of the basal layer. A similar staining pattern was also found by Smart's group (Oh and Smart, 1998). However, there is also some nuclear staining of C/EBPα in cells at the suprabasal layers (arrows in Fig 6.4 B). Therefore I postulate there might be an unknown post-transcriptional regulatory mechanism to prevent C/EBPα from entering the nucleus, probably exerting an inhibitory effect to its normal function at the basal stratified squamous gene expression.

According to the expression profile I find, C/EBPβ is more likely to activate the suprabasal differentiation genes in the oesophageal epithelium, such as *K1* and *K10*. Whether the transcription factor AP-2 is still involved in the regulation of the two C/EBP transcription factors remains to be examined.

6.1.3.4 p63

p63 is present from E11.5 in the basal layer of the oesophagus epithelium. The timing of expression is much earlier than K14 (which starts to be expressed around E15.5-E17.5 *in vivo* or 5-7 days of culture based on immunostaining). This means p63 is expressed at least 5-7 days before the stratified squamous basal cell fate is committed. It is unlikely that a transcription factor involved in directly regulating gene expression will take this amount of time to activate an immediate early gene. Either there are specific isoform(s) of p63 that are responsible for inducing K14 at the right time but which cannot be distinguished by RT-PCR or immunostaining methods; or, of course, *K14* might not be the immediate early target genes of p63.

One of the postulated functions of p63 is to commit epithelial cell fate to a stratified squamous tissue. Recently, two groups (using zebrafish as a model system) found that p63 is responsible for the initiation of stratified squamous tissues (including the formation of the epidermis and fin bud (equivalent to the limb in mammals)) (Bakkers et al., 2002; Lee and Kimelman, 2002). Interestingly, zebrafish only possesses the ΔNp63 isoforms but not the TAp63 isoforms. Using a luciferase assay, BMP2b has been demonstrated to be an upstream signal that induces ΔNp63 expression in zebrafish. Downstream effectors of BMP signals including SMAD4 and SMAD5 can directly bind to the *ΔNp63* promoter and activate the expression of the gene. BMP signalling is important in determining the ectodermal cell fate to become epidermis instead of neural cells in early development (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997a; Suzuki et al., 1997b), and this mechanism might be mediated through activation of ΔNp63 to suppress the neuronal program when determining the cell fate of the ectoderm to become epidermis.

In conclusion, I need more evidence to clarify the roles of these transcription factors in the induction and maintenance of the oesophageal stratified squamous tissue. In the next section, I will extend the study by assaying the activity of the *K14* and *K18* promoters in different cell lines when candidate transcription factors are introduced. Columnar epithelial cell lines will also be transfected with some of the candidate transcription factors and immunostained for ectopic K14 expression.

6.2 Analysis of candidate transcription factors on cell lines - Luciferase assay and stratified squamous metaplasia

6.2.1 Introduction

In order to determine the molecular mechanisms that control the change from a simple columnar to stratified squamous tissue, I used two approaches. The first was based on a reporter assay to quantify the changes in gene expression of columnar and stratified squamous genes in an *in vitro* cellular system. The second approach was to directly induce the transdifferentiation of columnar epithelia to a stratified squamous phenotype by the introduction of candidate transcription factor(s). Ideally, there might be a master switch gene that will simultaneously control down-regulation of the columnar genes, i.e. K8 and K18 at the basal layer and at the same time causes the up-regulation of the basal markers of a stratified squamous tissue (expression of K5 and K14).

6.2.1.1 Specificity of K18 and K14 promoters

In order to investigate how genes of the simple columnar and stratified squamous basal layer cells are regulated, I needed to establish biochemical evidence that the transcription factors can activate the stratified squamous specific *K14* gene and repress the simple columnar *K18* gene. To address this problem, I used a luciferase assay based on co-transfection of the transcription factor and the reporter gene (*K14* and *K18* promoter linked to an enhanced luciferase reporter, GL3 plasmid from Promega). The human *K14* cassette promoter construct for making the luciferase reporter was the same as the *K14-nucGFP* used in Fig 4.3. Vasioukhin et al. showed that the *K14* promoter was able to drive expression of lacZ in stratified squamous tissue such as skin, tongue, and the oesophagus (Vasioukhin et al., 1999). However, it is also

possible that there might be promiscuity of the promoter when it is transfected into cell lines *in vitro* (Vassar et al., 1989). To address this possibility, I have also carried out control electroporation experiments. The *K14* promoter construct driving nuclear GFP was electroporated into organs expressing a columnar phenotype such as embryonic stomach, intestine and lung cultures. I saw no cells expressing GFP (Fig 4.4 B-D). In contrast, GFP under the control of the *CMV* promoter was expressed in the tissues under the same experimental conditions, demonstrating that the electroporation is working (Fig 4.4 F, G). It appears that the *K14* promoter I use is faithfully expressed in the tested stratified squamous embryonic oesophagus after electroporation.

To study the down regulation of the columnar genes, I also used a *K18* promoter for luciferase reporter assay. The *K18* promoter construct has been engineered to contain the following elements: the 2.5 kb 5 upstream enhancer and promoter region of the human *K18* gene (which includes the minimal promoter region), the 1st intron of the K18 gene which has strong enhancer activity, plus a translation enhancer element (TE) inserted immediately 5 end of the start codon, the whole length of the *K18* promoter / enhancer regulatory construct consists about 3.2 kb in total (Koehler et al, 2001). The TE element was derived from the alpha1fa mosaic virus to enhance translation of the mRNA (Chow et al., 1997). The *K18* promoter is shown to consist of elements specific to the columnar specific tissue, in the *K18-LacZ* transgenic mouse, the promoter controls expression in places such as the airway epithelium of the bronchiole and lung (Chow et al., 1997; Koehler et al., 2001). The promoter will therefore be useful for the present study since the origin of oesophagus and trachea / bronchiole / lung are closely related during foregut development.

I have assayed for promoter activity using a luciferase assay. The assay is

based on the promoter activating or repressing the downstream reporter (in this case luciferase GL3). When an activator or repressor transcription factor binds to the promoter region, the change in fluorescence produced by the luciferase-substrate reaction can be quantified in the transfected cells. The luciferase vectors (under the control of the *K18* or *K14* promoter elements) are co-transfected with candidate transcription factors (*AP-2α*, *C/EBPα*, *C/EBPβ*, *TAp63α*, *TA*p63α* or *ΔNp63α*) into oesophageal or non-oesophageal cell lines. The experiments should provide evidence if any of the transcription factors are responsible for suppression of *K18* or induction of *K14* genes. In order to mimic the *in vivo* situation it is important to use oesophageal cell lines that recapitulate the normal expression pattern of keratin genes. I chose four cell lines representative of columnar, stratified squamous or mesenchymal cell types to investigate how the transcription factors act on the promoters in different cellular contexts (See Table 6.1 for experimental design).

6.2.1.2 Cell lines

The four cell lines I utilized to test the effect of candidate transcription factors on the promoters activities were (a) OE33, a human oesophageal carcinoma cell line, derived from a Barrett's patient (Jones et al., 2003), and express columnar cell markers. (b) KYSE30 is an oesophageal stratified squamous carcinoma cell line (Rigberg et al., 1998; Shimada et al., 1992). (c) NIH3T3 4.2, is a mouse fibroblast cell line (null control for columnar and stratified squamous keratin markers). However during the progress of the work I found that NIH3T3 cells express K14. (d) CaCO2, a human colon adenocarcinoma, is thought to be strictly columnar with no stratified squamous marker expression (Baricault et al., 1994; Fossar et al., 1999). I tried the *K14* and *K18* promoter luciferase

Triplicates	Cell types (4)	pCMV-nucLacZ (transfection control)	TFs (1 µg each)	Reporters (3)	Transfection duration
	CaCO2		pcDNA3 + pcDNA3	pK18mTELucE	48 h
	OE33		pC/EBPα + pcDNA3	pK14-LucE	
	KYSE30		pC/EBPβ + pcDNA3	pGL2	
	NIH3T3 4.2		pAP-2α + pcDNA3		
			pTAp63α + pcDNA3		
			pΔNp63α + pcDNA3		
			pTA*p63α + pcDNA3		
			pTAp63α+ pΔNp63α		
			pTA*p63α+ pΔNp63α		

Table 6.1 Luciferase assay.

assay on these different cell lines testing transcription factors – *AP-2α*, *C/EBPα*, *C/EBPβ*, *TAp63α*, *TA*p63α* and *ΔNp63α*. For analysis of the stratified squamous inducing function of the transcription factors - *AP-2* and *p63*s, I used Caco-2 and HepG2, a human hepatoma cell line that does not normally express stratified squamous markers (Morris et al., 1982).

From Chapter section 6.1, I learned which transcription factors express in the epithelium of the oesophagus during development. In the present chapter I used a luciferase assay to test the transcription factors and assay their effect on *K14* and *K18* promoters. I also directly transfected some of the transcription factors into columnar cells to examine their role in inducing the stratified squamous marker K14.

6.2.2 Results

6.2.2.1 Transcription factors detection after transfection

Before I could begin asking about the relationship between the transcription factors and the *K18* and *K14* promoters, I first needed to verify that the transcription factor constructs were being expressed and whether the proteins could be detected by the antibodies available in the lab. I therefore transiently transfected cells with plasmids expressing the transcription factors and then immunostained for the transcription factors. The transcription factor constructs were under the control of the ubiquitously expressed *Cytomegalovirus* (*CMV*) promoter. I transfected the following transcription factors: mouse *AP-2α*, rat *C/EBPα*, rat *C/EBPβ*, human *TAp63α*, human *TA*p63α* and human *ΔNp63α* into the *K14*-negative colonic cell line - Caco-2. The cells were cultured for two days after transfection. All the transcription factors were detected (Fig 6.6). However, the detection sensitivity for TA*p63 isoform (Fig 6.6 D, E cf. Fig 6.6 F), was not as great as the other transcription factors probably because the conformation is different with the additional N-terminal 39 amino acids.

6.2.2.2 Characterisation of cell lines and the luciferase promoter constructs

Before I quantified the *K14* and *K18* promoter activity by luciferase activation, I characterised the cell lines. I immunostained for K14 or K8 on each cell line to verify expression of the two keratin markers and compared with the detection of the *K14-GFP* and *K18-LacZ* after 2 days of transfection in each cell line (Fig 6.7).

K14 was found expressed in the oesophageal cell lines OE33 and KYSE30 (Fig 6.7 B, C), but not in the Caco-2 colonic cell line (Fig 6.7 A). This result showed that in the transformed adenocarcinoma cell line OE33, there are still

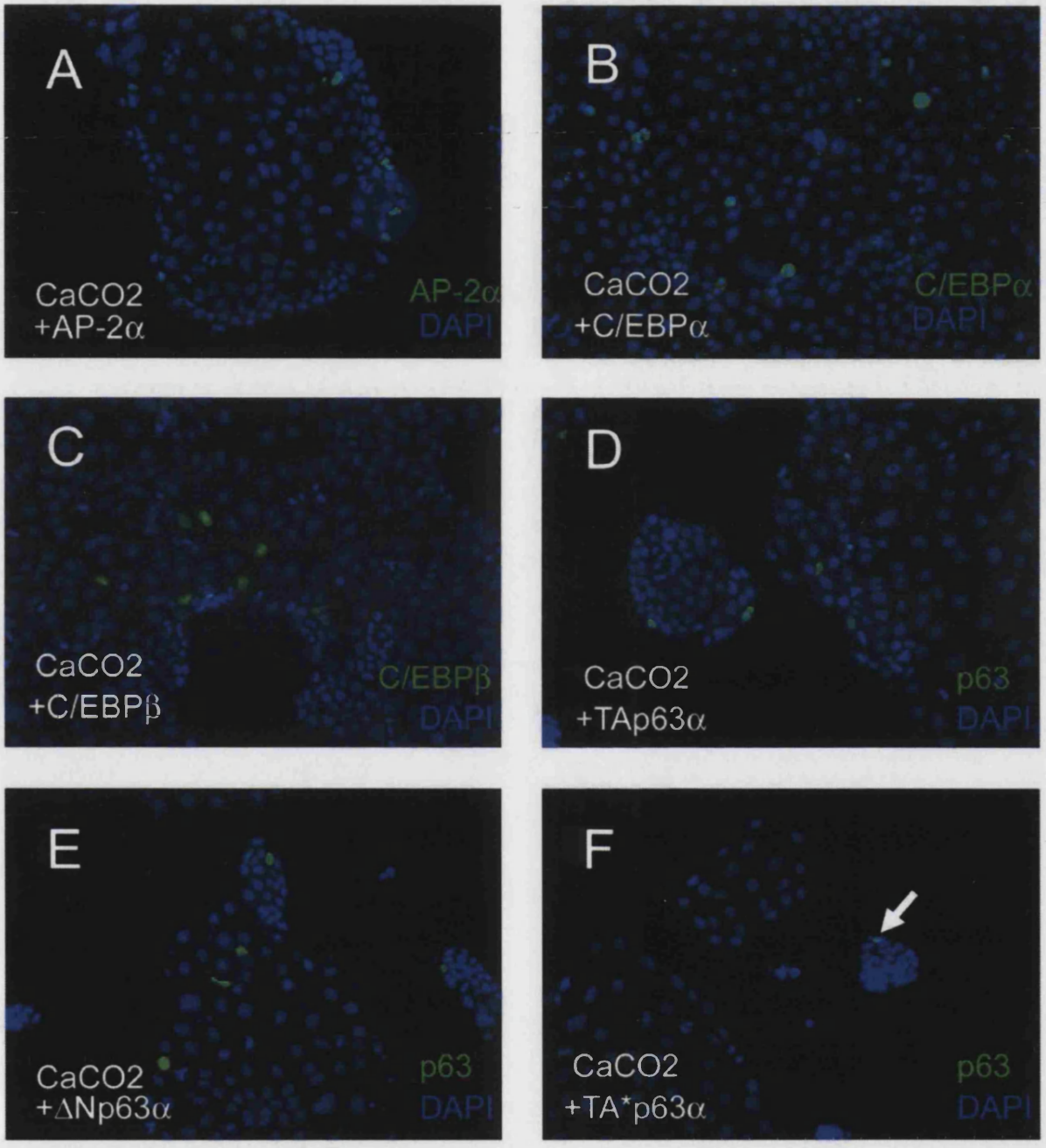

A
CaCO2
+AP-2α
AP-2α
DAPI
B
CaCO2
+C/EBPα
C/EBPα
DAPI
C
CaCO2
+C/EBPβ
C/EBPβ
DAPI
D
CaCO2
+TAp63α
p63
DAPI
E
CaCO2
+ΔNp63α
p63
DAPI
F
CaCO2
+TA*p63α
p63
DAPI

Fig 6.6 Ectopic expression of transcription factors in CaCO2 cells.

CaCO2 cells do not normally express stratified squamous transcription factors. Cells were transfected for 24 hrs with 1 μg transcription factors (*AP-2α* (A), *C/EBPα* (B), *C/EBPβ* (C), *TAp63α* (D), *ΔNp63α* (E) and *TAp63α* (F) and cultured for a further 48 hrs before fixation and immunostaining for AP-2α (A), C/EBPα (B), C/EBPβ (C) and pan-p63 (D-F). Transfection of transcription factors shows that both the full length gene constructs and the antibodies used are effective.

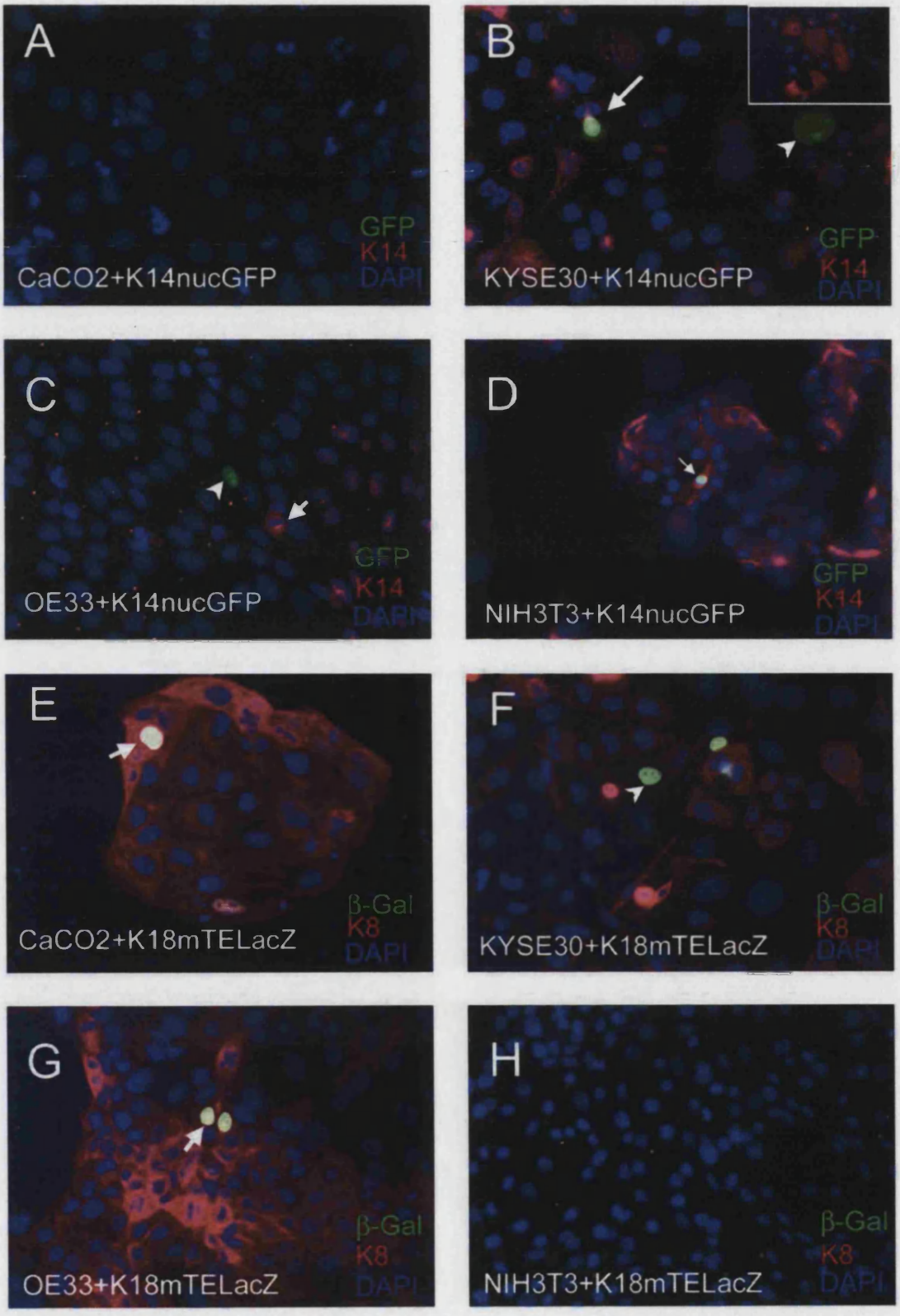

A
CaCO2+K14nucGFP
GFP
K14
DAPI
B
KYSE30+K14nucGFP
GFP
K14
DAPI
C
OE33+K14nucGFP
GFP
K14
DAPI
D
NIH3T3+K14nucGFP
GFP
K14
DAPI
E
CaCO2+K18mTELacZ
β-Gal
K8
DAPI
F
KYSE30+K18mTELacZ
β-Gal
K8
DAPI
G
OE33+K18mTELacZ
β-Gal
K8
DAPI
H
NIH3T3+K18mTELacZ
β-Gal
K8
DAPI

Fig 6.7 K8 and K14 expression.

Four cell lines were tested for expression of K14 and K8 by antibody staining alongside reporter gene expression driven by *K14* or *K18* promoters. The *K14* promoter driving GFP (A-D) and the *K18* promoter driving LacZ (E-H) were transfected into cell lines: CaCO2 (A, E), KYSE30 (B, F), OE33 (C, G) and NIH3T3 4.2 (D, H). Arrows show co-expression of the keratin protein expression with the GFP or β-gal reporters in the same cells (B, D, E, G). Arrowheads show the reporter expressed alone without the related keratin gene expression (Fig 5.8 B, C and F). Inset in B showing many KYSE30 cells expressing K14. Arrow in C shows K14 expression in one OE33 cell.

K14-positive cells although they appear less frequently than in the KYSE30 stratified squamous carcinoma line (Fig 6.7 B cf. arrow in C). I also found abundant expression of K14 in NIH3T3 cells (Fig 6.7 D). The expression of epithelial keratins in cultured fibroblastic cell lines was also observed by other groups (Katagata et al., 2002).

In *K14-nucGFP* transfected cell lines, GFP was expressed in OE33, KYSE30 and NIH3T3, but not in Caco-2 cell lines, consistent with the results of K14 protein expression. I found examples of cells co-expressing nuclear GFP (green) with keratin 14 (red) (arrows in Fig 6.7 B and D). However, on more frequent occasions, I found nuclear GFP in cells that do not express K14 (arrow heads in Fig 6.7 B, C). These results suggest that the promoter activity is not totally equivalent to the endogenous keratin expression in the cell lines.

K8 was detected in Caco-2 cells (Fig 6.7 E), in oesophageal adenocarcinoma OE33 (Fig 6.7 G) and in the stratified squamous carcinoma KYSE30 cell lines (Fig 6.7 F), but not in NIH3T3 fibroblasts (Fig 6.7 H). The *K18* promoter driving nuclear LacZ showed a stronger correlation to the protein expression compared to the *K14* promoter. The nuclear lacZ expression was found in Caco-2 (Fig 6.7 E) and OE33 cells (Fig 6.7 G) co-expressing K8 protein, but there were also KYSE30 cells in which the K8 proteins expression is absent in cells expressing nuclear LacZ (arrowheads in Fig 6.7 F). It therefore seems that the promoters I use behave differently in different cellular contexts. A combined analysis of results obtained in these diverse conditions will likely yield more clues on the regulation of keratin promoters.

6.2.2.3 Luciferase assays

I examined the effect of individual transcription factors (*AP-2α*, *C/EBPα*,

C/EBPβ and *TA*, *ΔN* and *TA* p63α* isoforms alone and in combination) on the columnar *K18* promoter-*K18mTELucE* (Fig 6.8) and the stratified squamous promoter *pK14-Luc* (Fig 6.9) (Maps of the constructs can be found in Appendix A-1). The idea of testing the combined effect of transcription factors arises from the observation that different p63 isoforms could differentially affect the initiation of stratified squamous epithelium (Koster et al., 2004). There might also be a different effect to the tested promoters when the TA isoform and the ΔN isoform are present together, e.g. they might be antagonistic.

For each assay I transfected 1 μg of the promoter luciferase reporter constructs along with 1 μg of a *pCMV-nucLacZ* plasmid and 1 μg of each transcription factor (see Table 6.1 for details). Whenever a single transcription factor was being tested, I added 1 μg of empty pcDNA3 plasmid. The results shown were from transfections carried out three times and each transfection sample was assayed four times. The relative luciferase unit (RLU) is normalized by the total protein loaded, and the transfection efficiency is normalized by a colorimetric measurement of the β-Gal activity from the *pCMV-nucLacZ* plasmid.

In terms of the effect of *AP-2α* or *C/EBPα* on the *K18* promoter-*K18mTELucE*, my results showed no significant difference in the promoter activity of the two transcription factors compared to the pcDNA3 control in all four cell lines (Fig 6.8 A, B). *C/EBPβ* in KYSE30 was the most promising candidate as a repressor of the *K18* promoter (0.39±0.05 of the K18 promoter activity of the control pcDNA3) (Fig 6.8 C). However, *C/EBPβ* had a tendency to act as an activator instead of a repressor in other cell lines tested (Fig 6.8 C), although the activator activity in compared to the control is not statistically significant.

For the *TAp63α* and *ΔNp63α* effect on the *K18* promoter, there is a slight

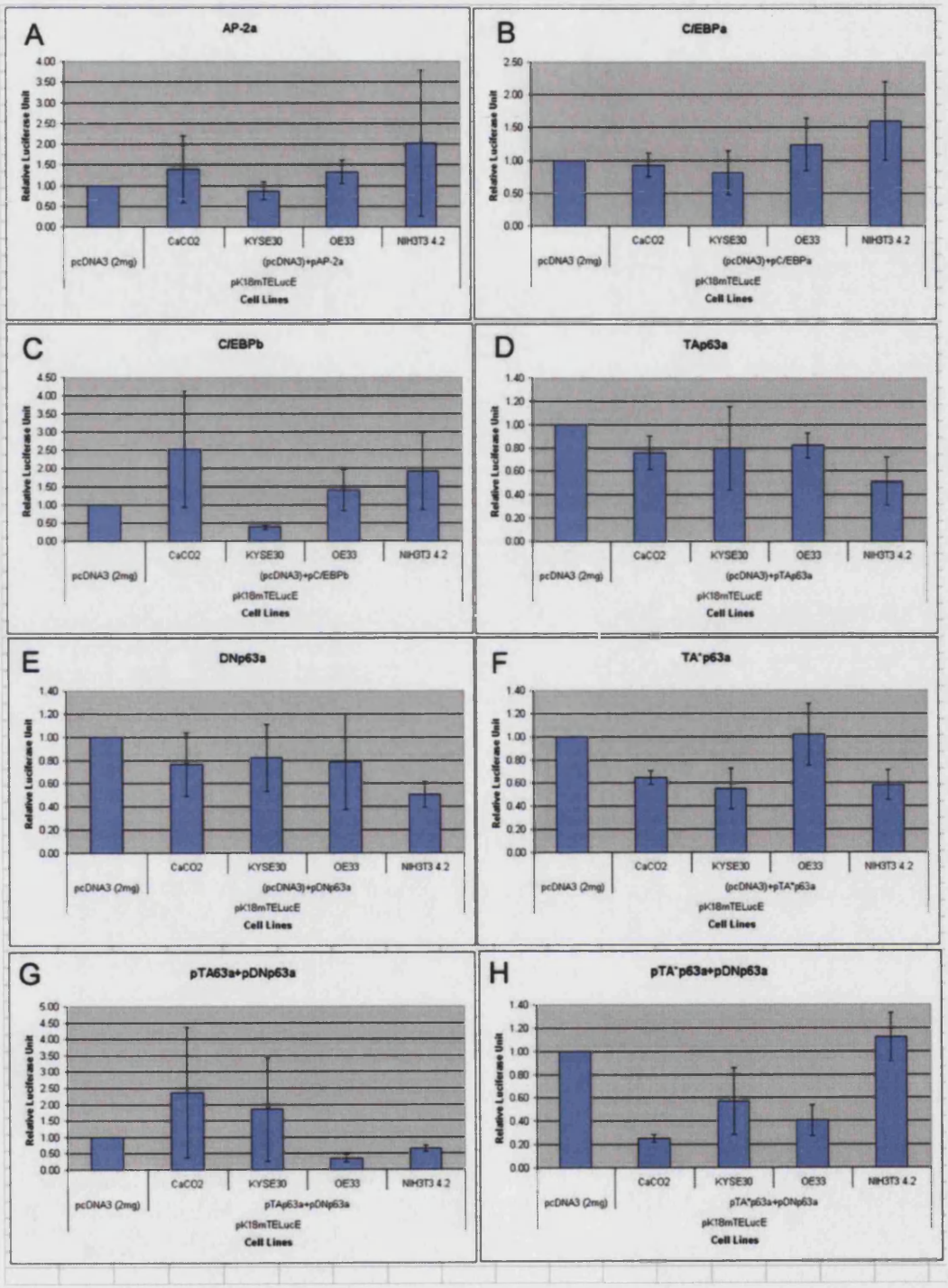

A
AP-2a
B
C/EBPa
C
C/EBPb
D
TAp63a
E
DNp63a
F
TA*p63a
G
pTA63a+pDNp63a
H
pTA*p63a+pDNp63a
Relative Luciferase Unit
pcDNA3 (2mg)
CaCO2
KYSE30
OE33
NIH3T3 4.2
(pcDNA3)+pAP-2a
(pcDNA3)+pC/EBPa
(pcDNA3)+pC/EBPb
(pcDNA3)+pTAp63a
(pcDNA3)+pDNp63a
(pcDNA3)+pTA*p63a
pTAp63a+pDNp63a
pTA*p63a+pDNp63a
pK18mTELucE
Cell Lines

Fig 6.8 *K18* promoter activity in cell lines.

Luciferase under the control of the *K18* promoter (Koehler et al., 2001) was assayed in four different cell lines (Caco-2, KYSE30, OE33 and NIH3T3), by co-transfection with 1 μg of transcription factor and 1 μg of control pcDNA3 plasmid or a combination of 2 transcription factors (2 μg in total). 1 μg of β-Gal was transfected along with the transcription factors and the luciferase expressing plasmids for transfection control, and total protein assay was used as a loading control. The experiments were repeated 3 times.

repression in all the cell lines examined (Fig 6.8 D, E), and the effect of *TA*p63α* is similar, except in the OE33 cell line (Fig 6.8 F).

TAp63α and *ΔNp63α* in combination activates *K18* in Caco-2 or KYSE30 cell lines but represses *K18* in OE33 or NIH3T3 cells (Fig 6.8 G). *TA*p63α* and *ΔNp63α* in combination had the same effect on the *K18* promoter as alone (Fig 6.8 H).

For *K14* promoter luciferase assay, *AP-2α* mildly enhanced the promoter activity of *K14* in all cell lines (Fig 6.9 A), although this was less than reported previously (Byrne et al., 1994), the quantitative difference may be due to differences in the promoter length (I used 2,000 bp vs the published 2,300 bp). *C/EBPα* is slightly repressive on *K14* promoter activity except on NIH3T3 cells (Fig 6.9 B) whereas *C/EBPβ* activates *K14* promoter in Caco-2 or NIH3T3, but represses *K14* activity in KYSE30 or OE33 cell lines (Fig 6.9 C). *TAp63α* and *ΔNp63α* can activate the *K14* promoter activity in Caco-2 cell line, and the ΔN isoform has more *K14* promoter activating potential than TA isoform (Fig 6.8 D, E), although the ΔN isoform is repressive of K14 activity in OE33 (Fig 6.9 E). *TA*p63α* isoform also activated *K14* in Caco-2 cells, but not significantly, and was repressive in the OE33 cell line (Fig 6.9 F). The combination of the *TA* and *ΔN p63α* or *TA** and *ΔN* isoform activated *K14 promoter* (4.2x and 3.8x, respectively compared to the pcDNA3 control) in Caco-2 cells (Fig 6.9 G, H), although there is some variations in the results. The effect of combined transcription factors (*TA* or *TA** isoform with *ΔN* isoform) is repressive to the *K14* promoter activity in other cell lines (Fig 6.9 G, H).

In conclusion, the relative luciferase (RLU) results of the luciferase assay showed wide variations. The variation may arise from the amount of DNA used (4 μg in total including 1μg of β-Gal construct, 1 μg of luciferase reporter and

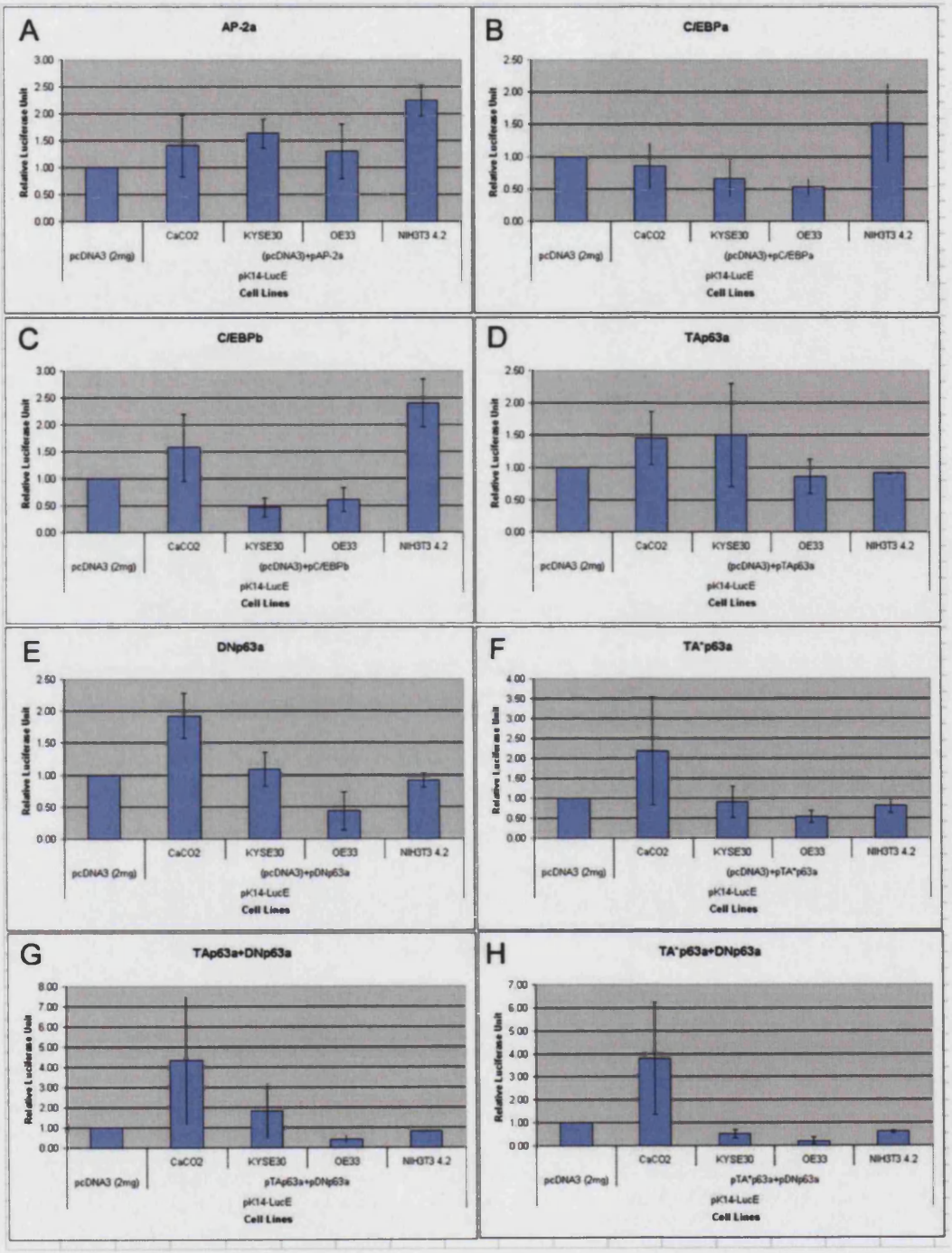

A
AP-2a
B
C/EBPa
C
C/EBPb
D
TAp63a
E
DNp63a
F
TA*p63a
G
TAp63a+DNp63a
H
TA*p63a+DNp63a
Relative Luciferase Unit
pcDNA3 (2mg)
CaCO2
KYSE30
OE33
NIH3T3 4.2
(pcDNA3)+pAP-2a
(pcDNA3)+pC/EBPa
(pcDNA3)+pC/EBPb
(pcDNA3)+pTAp63a
(pcDNA3)+pDNp63a
(pcDNA3)+pTA*p63a
pTAp63a+pDNp63a
pTA*p63a+pDNp63a
pK14-LucE
Cell Lines

Fig 6.9 *K14* promoter activity in different cell lines.

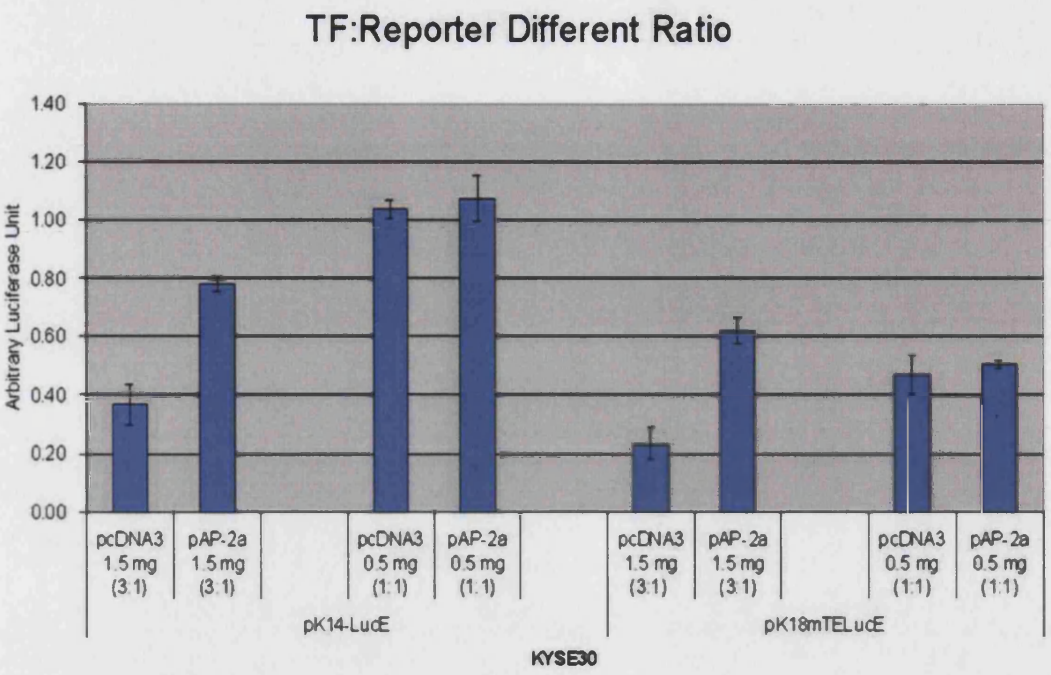
K14 promoter (Vasioukhin et al., 1999) activity was assayed in four different cell lines (Caco-2, KYSE30, OE33 and NIH3T3), after co-transfection with 1 μg of transcription factor (and 1 μg of control pcDNA3 plasmid) or combination of 2 transcription factors (2 μg in total). 1 μg of β-Gal plasmid was transfected along with the transcription factors and the luciferase expressing plasmids for transfection control, and total protein assay were used as a loading control. The experiments were repeated 3 times.

2 µg of transcription factors). It is possible I saturated the cell transcription and translation machinery. There might also be a difference in the luciferase assay results on the effect of different molar ratio of transcription factor vs luciferase reporter (TF: Luc) (see Fig 6.10) Therefore, I set out to perform a control transfection experiment using less DNA and also to test the effect of different molar ratios of transcription factor vs luciferase reporter.

Instead of using 1 µg of transcription factor or luciferase construct, I used 0.5 µg of the luciferase reporter and either 0.5 or 1.5 µg of the transcription factor. The β-Gal transfection control was still 1µg. I decided to test *AP-2α* on both *K18* and *K14* promoters in KYSE30 cells since it was already known that *AP-2α* can directly bind to and activate the *K14* promoter (Sinha et al., 2000). The *AP-2α* effect on the *K14* promoter is 2.1 fold more active than the pcDNA3 control when the TF:Luc is 3:1, but there is no difference when the TF:Luc is 1:1 (Fig 6.10). The effect on *K18* is 2.6 fold more active than the pcDNA3 control when the TF:Luc is 3:1, and also no difference when the TF:Luc is 1:1. The results in Fig 6.10 have a much smaller standard error bar comparing to Fig 6.8 and Fig 6.9. Using less DNA provides a more consistent result indicating the importance to titrate the DNA amount before commencing the experiments.

6.2.2.4 Ectopic expression of the candidate transcription factors in Caco-2 and HepG2

In order to test the idea that some of the candidate transcription factors might induce the expression of stratified squamous markers in cell lines that are not stratified squamous epithelia in origin, I transfected *AP-2α*, *TAp63α*, *ΔNp63α* and *TA*p63α* into Caco-2 and HepG2 cell lines. *AP-2α* transfected alone


TF:Reporter Different Ratio
Arbitrary Luciferase Unit
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
pcDNA3 1.5 mg (3:1)
pAP-2a 1.5 mg (3:1)
pcDNA3 0.5 mg (1:1)
pAP-2a 0.5 mg (1:1)
pcDNA3 1.5 mg (3:1)
pAP-2a 1.5 mg (3:1)
pcDNA3 0.5 mg (1:1)
pAP-2a 0.5 mg (1:1)
pK14-LucE
pK18mTELucE
KYSE30

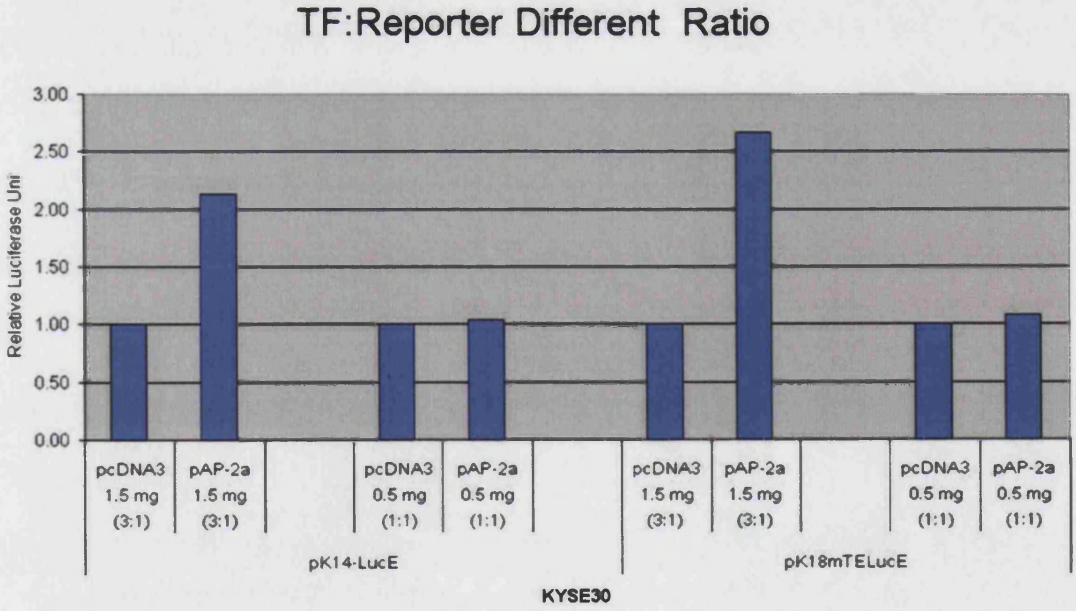

TF:Reporter Different Ratio
Relative Luciferase Uni
3.00
2.50
2.00
1.50
1.00
0.50
0.00
pcDNA3 1.5 mg (3:1)
pAP-2a 1.5 mg (3:1)
pcDNA3 0.5 mg (1:1)
pAP-2a 0.5 mg (1:1)
pcDNA3 1.5 mg (3:1)
pAP-2a 1.5 mg (3:1)
pcDNA3 0.5 mg (1:1)
pAP-2a 0.5 mg (1:1)
pK14-LucE
pK18mTELucE
KYSE30

Fig 6.10 The ratio of transcription factors to luciferase reporter influences the promoter luciferase assay.

1.5 μg of the transcription factor *AP-2α* transfected with 0.5 μg of the *K14* or *K18* promoter controlled luciferase give different luciferase results to 0.5 μg of the *AP-2α* transfected with 0.5 μg of the reporters.

cannot induce the ectopic expression of K14 in Caco-2 or HepG2 cells (results not shown). This result confirmed the observation by Fuchs group who also transfected AP-2 into HepG2 and did not find K5 or K14 expression (Leask et al., 1991).

When *p63* was transfected into Caco-2 (Fig 6.11 A-D) or HepG2 (Fig 6.11 F-I) cells for 5 days, K14 became expressed. Interestingly, both the ΔN (Fig 6.11 A, B, F, G) and the TA (Fig 6.11 C, D, H, I) isoforms showed ectopic K14 expression but the TA* isoform did not (results not shown). When examined under lower magnification, transfection of the ΔN isoform actually gave rise to more K14 positive cells compared to the TA isoform (Fig 6.11 K cf Fig 6.11 L). In HepG2 cells, I observed seven K14 positive cells in a single field where there are six cells in the same field showing p63 expression after the ΔN isoform transfection (Fig 6.11 K). While there is only one K14 positive cell in a similar field of HepG2 cells which showed nine p63 expressing cells under the same condition of TA isoform transfection (Fig 6.11 L). Similar differences are found for the ΔN and TA isoforms in Caco-2 cells (results not shown). I also observed K14 positive cells where no p63 is detectable (arrows in Fig 6.11 C, K, L). This is found in both ΔN and TA isoform transfection, but no K14 positive cells are found when the columnar cell lines were transfected with a GFP control plasmid (Fig 6.11 E, J).

A
Caco-2+ΔNp63α 5d
K14
p63
DAPI
400x
B
400x
C
Caco-2+TAp63α 5d
K14
p63
DAPI
400x
D
400x
E
Caco-2+nucGFP 5d
GFP
K14
DAPI
400x
F
HepG2+ΔNp63α 5d
K14
p63
DAPI
400x
G
400x
H
HepG2+TAp63α 5d
K14
p63
DAPI
400x
I
400x
J
HepG2+nucGFP 5d
GFP
K14
DAPI
400x
K
HepG2+ΔNp63α 5d
K14
p63
DAPI
100x
L
HepG2+TAp63α 5d
K14
p63
DAPI
100x

Fig 6.11 *p63* induces expression of *K14* in columnar cell lines.

1 µg of transcription factor *ΔNp63α* (A-D, I) or *TAp63α* (E-H, J) was transfected into two columnar cell lines – Caco-2 (A, B, E, F) and HepG2 (C, D, G, H, I, J). *K14* positives cells are found 5 days after transfection of both *p63* isoforms and in both cell lines. However, lower magnification of HepG2 transfected with the two isoforms showed that there are more K14 positive cells in the *ΔNp63α* than the *TAp63α* transfected cells. Some cells express K14 without p63 expressing in the nucleus (Arrows in E, I, J).

6.2.3 Discussion

6.2.3.1 Luciferase results

From the results of the luciferase assay, it is not easy to draw any substantive conclusions on the role of transcription factors either as activators or repressors for the same promoter in different cell lines. Similar results were also found for C/EBPα or C/EBPβ. These transcription factors act as repressors of *α2* and *α5-integrin* promoter constructs in primary human keratinocytes, but act as activators of *α2* and *α5-integrin* promoter in the human hepatoma cell line-HepG2 (Corbi et al., 2000). Until now, no one has examined the effect of C/EBPα and C/EBPβ on the *keratin 14* and *keratin 18* promoters, although they were found to regulate the *K1* and *K10* (suprabasal) genes (Maytin et al., 1999). However, in light of the results from the experiment using different amounts of DNA and different TF to Luc ratio, future work might start by optimizing the assay before I can interpret the promoter activities (Oh and Smart, 1998).

It is surprising that the p63 isoforms do not have more of an impact on K14 activity on OE33 and KYSE30 oesophageal cell lines. From the *p63* knockout studies and the observation that the TAp63 isoform initiates epidermal stratified squamous tissue (Yang et al., 1999; Mills et al., 1999; Koster et al., 2004), it would be expected that the TAp63 isoform should activate *K14* promoters. In this context, it would be of interest to determine the relationship between K14 expression and p63. It is surprising as to why no-one has studied the *K14* promoter activity and p63 or direct binding analysis of p63 and *K14* promoter. One possibility that I do not find significant activation of K14 by p63 is that the *p63* binding element is not on the *K14* and *K18* promoter constructs used in the present study. This would mean the enhancer element responsible

for p63 binding is not located in the 2.0 kb bp or 3.2 kb regulatory sequence in the *K14* and *K18* promoter constructs, respectively. Another possibility is that p63 does not directly bind to *K14* and/or *K18* promoter enhancer region at all, but is acting through other upstream factor(s) regulating initiation of the stratified squamous programme. This would mean I might observe significant activation of the *K14* promoter if I leave the transfected cells cultured for longer periods of time. There are currently no consensus *p63* binding sites known, however, there are studies showing p63 might bind to *p53* binding elements (Yang et al., 2002). From my preliminary results using bioinformatics for consensus binding elements for transcription factors, both human *K14* and *K18* promoter/enhancer regions contain *p53*-like binding sites (data not shown), but unless I perform an electromobility shift assay (EMSA) or chromatin immunoprecipitation (CHIP) with antibodies specific to the p63 transcription factor, there is still no way to tell whether the p63 isoforms bind directly to the *K14* promoter.

6.2.3.2 Ectopic expression of the candidate transcription factors in columnar cell lines

ΔNp63α and *TAp63α* but not *AP-2* or *TA*p63α* have the ability to activate K14 expression in Caco-2 and HepG2 columnar cell lines. This result suggests that members of the p63 family may act as master switch gene(s) inducing the formation of stratified squamous epithelium. In addition, knowing that *ΔNp63α* and *TAp63α* as potential master switch genes, they might have therapeutic potential to reverse stratified squamous abnormalities such as Barrett's metaplasia.

Three lines of evidence obtained in the present study suggest that *ΔNp63*

isoform is important in the initial formation of stratified squamous epithelium instead of *TAp63* isoform as previously suggested (Koster et al., 2004). This includes (a) expression analysis by RT-PCR (Fig 6.2), which demonstrates that the *ΔNp63* isoform is expressed earlier than both the *TAp63* and *TA** isoform, (b) luciferase analysis in Caco-2 cells shows that the ΔN isoform induces stronger *K14* promoter activity than the TA isoform, and (c) by transfection studies, the ΔN isoform induces more K14 positive cells than the TA isoform. However, both ΔN and TA isoforms can induce *K14* expression in the luciferase assay and the transfection study, suggesting there might be a positive (auto-)regulatory feedback loop of each of the isoforms to induce one another. Evidence for this hypothesis is that putative *p63* binding elements were also found in the enhancer region of the *ΔN p63* gene (Yang et al., 2002). One curious observation is some of the cells were found expressing K14 but not p63 after *p63* transfection. There are at least two possibilities: (1) the need for p63 to induce K14 is temporary. Therefore, there might be cells that had lost p63 expression, but the *K14* gene is already activated and (2) there might be paracrine signalling from p63 positive cells to p63 negative cells instructing them to start expressing K14. There are of course possibilities other than these two explanations such as the existence of a *p63*-independent pathway for K14 expression, but I currently have no means of testing.

Future work for understanding *p63* function is to test other stratified squamous differentiation markers such as K10 and involucrin in *p63*-transfected cells. I would hope to find the full repertoire of stratified squamous genes expressed in the transfected columnar cells if *p63* is truly a master switch gene for stratified squamous transdifferentiation. In addition, other nuclear factors such as *AP-2* and members of the *C/EBP* family might still be required to complete the full

transdifferentiation process.

Chapter 7 Conclusions, Discussions and Future Work

7.1 Summary

The development of the oesophagus has not received much academic attention. The reason is not clear but in part may be the fact that it appears relatively simple with no intricate cellular and functional complexity. Moreover, up until now, there has been no suitable *in vitro* system for studying its development and function. The oesophagus is unique in the sense that it is the only endodermally-derived organ that contains stratified squamous epithelium (Wells and Melton, 1999). In the current thesis, I have examined the development of the oesophagus and in particular, focused on the formation of the stratified squamous epithelium.

There are three types of cell type conversions that arise either during development of the oesophagus or in certain human oesophageal pathologies. These conversion are (a) the change from simple columnar to stratified squamous epithelium (at around embryonic day 15.5 to 17.5 in mice), (b) a change from the smooth muscle to skeletal muscle during the perinatal period and (c) conversions of stratified squamous to columnar epithelium in the clinical condition - Barrett's metaplasia. Barrett's metaplasia predisposes to development of adenocarcinoma (Paulson and Reid, 2004). The rising incidence of Barrett's metaplasia and oesophageal adenocarcinoma in Western countries has led researchers to search for the underlying mechanism(s) as well as a cure for the disease. Based on the clinical importance of Barrett's metaplasia, researchers have also come to realise the importance in understanding how endodermal organs are formed during development, and to identify the molecular and cellular differences between

organs such as the oesophagus and intestine.

In Chapter 3, I studied the normal development of the mouse embryonic oesophagus from stage E11.5. After E10, the foregut has separated into two lumen structures, the oesophagus and the trachea / lung buds (Motoyama et al., 1998). My study is the first to systemically characterise in detail the morphological appearance of the oesophagus as well as the muscular and epithelial markers of the developing mouse oesophagus throughout developmental stages from E11.5 to postnatal 2 months. I found that the cell type changes from smooth to skeletal muscle tissue (in agreement with a number of published studies) and also characterised in detail the conversion from columnar to stratified squamous epithelium in the oesophagus.

In addition to characterising the switch of epithelial phenotype *in vivo*, I also established an *in vitro* culture model for the oesophagus, which was based on isolation of mouse oesophagi from E11.5 embryos and cultured on fibronectin-coated dishes. The *in vitro* model recapitulated the *in vivo* switch from smooth to skeletal muscle and from columnar to stratified squamous epithelium. By using the oesophagus culture model, I could monitor the phenotypic changes more closely, and in addition, the model provides us with a means to manipulate the normal development of the oesophagus and to address a number of specific questions. I first compared the cultured oesophagi to *in vivo* oesophagus development, in which I used cytoskeletal and epithelial markers - K14, K8, K18, K4, K13, K10, involucrin and E-cadherin for characterising the epithelial tissue. I used markers such as desmin, smooth muscle actin, myoglobin and skeletal myosin for characterising muscle. The cell types for stratified squamous epithelium and oesophageal muscle cells were found in a similar spatial relation in culture compared to *in vivo*. From the

outer to the inner layers, there were fibroblastic cells, muscle layers, mesenchymal submucosal layers, basement membrane, basal layer and the suprabasal layers of stratified squamous epithelium (illustrated in Fig 1.4).

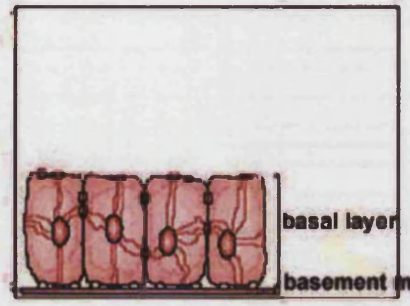
We were interested to know if the cells of the basal stratified squamous epithelium come directly from the simple columnar cells lining the embryonic oesophagus. I tried several methods and confirmed the idea that the stratified squamous tissue arise from the simple columnar epithelium. It is also interesting to examine if there is a requirement of cell division during the switch. I found that inhibition of cell proliferation, however, does not affect the transition from simple columnar to stratified squamous.

It is evident that the oesophagus does not need to interact with cells from other organs in controlling the epithelium to undergo a phenotypic change from columnar to stratified squamous in my culture system. Furthermore, reducing the thickness of mesenchymal layers accelerates this switch, suggesting there might be a repressive signal coming from the mesenchyme to suppress the switch, and the signals were gradually nullified during development. We hypothesised that either the switch is caused by (a) the columnar cells disappearing by apoptosis and there is an outgrowth of the stratified squamous epithelial progenitor cells, or (b) it is simply shutting off of the columnar genes. From the results in Chapter 4, we favour the second model. There is no significant difference between the number of dead columnar cells and dead stratified squamous cells *in vitro*, and inhibition of programmed cell death does not have any effect on the switch. Interestingly, inhibition studies with 5-aza-2-deoxycytidine found that DNA methylation might be responsible for silencing of the columnar genes.

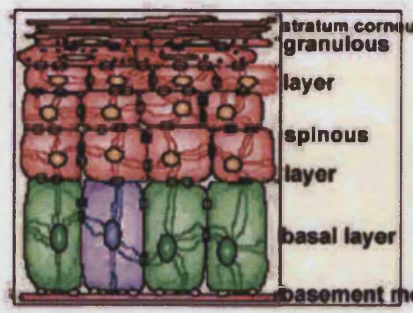
A number of transcription factors have been previously identified that might be

important for initiation of stratified squamous epithelium development. For example, different p63 isoforms are thought to control the induction of stratified squamous epithelium (Koster et al., 2004). In Chapters 6 I found that the ΔN isoform is expressed from E11.5 and the transcription factor was still expressed in the adult oesophagus. *p63* (probably the ΔN isoform) might act as a master switch gene to transdifferentiate colonic and hepatic cell lines to express stratified squamous (K14) markers. The cell transfection results also confirmed the results of the *K14* promoter/enhancer activity activated in the columnar cell line by ΔNp63α (Fig 6.9 E and F).

In conclusion, the work of this thesis provides a detailed description of the normal development of the mammalian oesophagus. Specifically, I described the morphological, cellular and molecular basis of oesophageal epithelial development. The results are summarised in Fig 7.1.

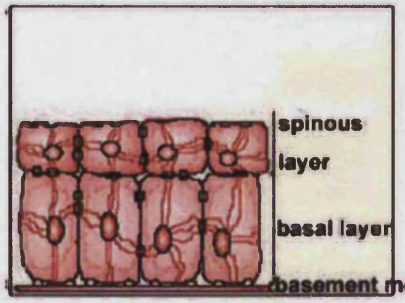


in vivo: E11.5
in vitro: E11.5+1d



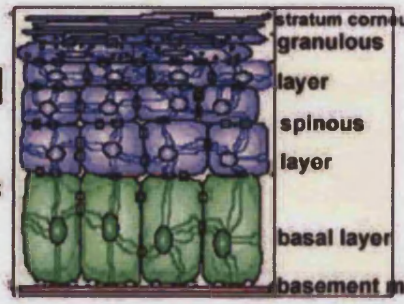
in vivo: P1 - P5
in vitro:E11.5+ 7-11d

suprabasal differentiation markers: K10 starts to express

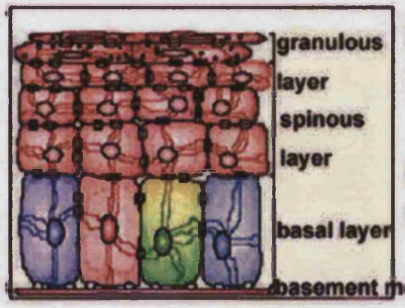


in vivo: E13.5-15.5
in vitro:E11.5+3-5d

K4 / K13 starts to express



in vivo: > P5 - adult
in vitro: E11.5 + 11d
- >15d



in vivo: E15.5-17.5
in vitro:E11.5+ 5-7d

Involucrin starts to express

: **K8/K18 positive cells**

: **K8/K18 negative cells (Dnmt3 ?)**

: **Converting cells (ΔNp63 ?)**

: **K14 positive cells**

Fig 7.1 A model for the conversion of oesophageal epithelium from simple columnar to stratified squamous tissue.

Summary for the development of stratified squamous in the oesophageal tissue. Comparative results are shown for both normal development and *in vitro* culture. At E11.5d or 1 day of culture, the oesophageal epithelium is only 1-2 cell layers thick and consists only of K8 positive cells. At E13.5-E15.5 (approximately 3-5 days of culture), the epithelium of the oesophagus becomes thicker, the submucosal and muscle layers are more defined, and keratin 4 is expressed (not shown). At E15.5-E17.5 (5-7 days of culture), the columnar K8 expression is lost at the basal layer and some basal cells start to express K14. In addition, involucrin starts to be expressed. In some segments of the oesophagus, we see epithelium that is characteristic of a granular layer near the lumen appear. At P1-P5 (7-11 days of culture), the basal layer of the epithelium is mostly K14 positive, but K8 positive cells are still retained in the suprabasal layers. We also see stratified squamous suprabasal differentiated marker K10 expression and very thin cornified layers. At adult (> 2 months old) (11 - >15 days of culture), K8 cannot be found in both the basal and suprabasal layers of the epithelium, and the oesophagus is fully differentiated as a stratified squamous tissue. *Dnmt3* may be responsible for the K8 silencing and *ΔNp63* may be involved in K14 induction.

7.2 Discussions and Future work

7.2.1 Oesophageal and foregut specific markers

My intention was to investigate development of the oesophageal epithelium. However, the lack of oesophageal specific markers somewhat hindered the study. We looked for stratified squamous markers in the epidermis to help characterise the oesophageal epithelium. The keratins K4 or K13 were used as regional markers for the oesophagus, since other organs that express these markers are tongue and cornea. We can distinguish the oesophagus from the neighbouring trachea/lung, stomach and intestine by using K4 and K13 antibodies (Moll et al., 1982). One question arising from this work is: are there known foregut markers expressed in the early oesophagus? *HNF3/forkhead* genes are members of the winged helix DNA binding domain family of transcription factors. *GATA4* and *GATA6* transcription factors contain distinct zinc finger DNA binding domains and are expressed in and are important for both the specification and maintenance of foregut identity (Zaret, 1999). To date, we have not looked closely at the expression of these transcription factors but in the future some attempt should be made to identify novel foregut and early oesophageal transcription factors. By conducting studies such as microarray analysis of the genes expressed during oesophageal developmental should facilitate (i) the identification of oesophagus-specific genes and (ii) the essential steps in organogenesis of the oesophagus.

7.2.2 Bifurcation and stratification

There are a number of interesting questions on oesophagus development that remain unanswered. For example, which factor(s) control the identity of the neighboring dorsal oesophagus and ventral trachea and lung? It is known from

knockout studies that Shh and the transcription factors *Nkx2.1* and *Foxf1* are important in bifurcation process of the foregut (Litingtung et al., 1998; Motoyama et al., 1998; Minoo et al., 1999; Mahlapuu et al., 2001). How does Shh actually regulate the bifurcation process is not clear? Since Shh is expressed in the gut endoderm (except in the pancreas (Roberts, 2000)), there must be other factors controlling invagination and closure of the foregut into two tube-like structures. However, bifurcation and stratification do not seem to be related, as an immature stratified squamous epithelium is still found on the dorsal side of the oesophageal-trachea fistula in the $Shh^{-/-}$ knockout mice (Litingtung et al., 1998). Inhibiting Shh with cyclopamine (Chapter 5, Fig 5.2), after bifurcation of the oesophagus and trachea/lung does not affect the induction of stratified squamous epithelium. This result confirms the notion that Shh might only be important for bifurcation but not related to the formation of stratified squamous epithelium in the oesophagus.

7.2.3 Signalling pathways involved in stratified squamous epithelium initiation

There are probably a number of signalling pathways essential in the maintenance of the adult oesophageal stratified squamous epithelium. For example, Ca^{2+} signalling has been shown to be important in controlling differentiation of stratified squamous epithelium (Green and Watt, 1982; Ma and Sun, 1986). We were interested in the early induction of the stratified squamous epithelial marker *K14*. I tried perturbing Wnt and Shh signalling, both signalling pathways were found to function in early foregut and oesophagus formation (Litingtung et al., 1998; Motoyama et al., 1998; Lickert et al., 2001; Theodosiou and Tabin, 2003). However, no suppression of *K14* expression was found either following activation of the Wnt / β-catenin pathway

by adding LiCl or suppression of Shh signalling by adding cyclopamine to the oesophagus culture.

Induction of ectoderm to either a neural or epidermal cell fate is determined by Bmp signalling (Hemmati-Brivanlou and Melton, 1997). During early gastrulation, Bmp is expressed along the primitive ectoderm. Noggin, a secreted inhibitor of Bmp, is found expressed where neurons are later formed. The ectodermal region where Bmp signalling is not inhibited, is induced to form epidermis (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997a). Recently, a zebrafish study of *ΔNp63* (the only form of *p63* in zebrafish), showed a requirement for p63 in the formation of the dermis and pectoral fin (equivalent to limbs in mice) (Bakkers et al., 2002; Lee and Kimelman, 2002). What is particularly exciting about this study was that *Smad* binding sites were found in the promoter/enhancer region of *ΔNp63*. Smad is presumably directly activating *ΔNp63* expression (Bakkers et al., 2002). *ΔNp63*, (like Bmp) is also suppressing the ventral neural fate in the zebrafish. These observations suggest that one of the upstream signals controlling *p63* is Bmp. It would be interesting to determine if Bmp or other TGFβ family members are involved in the up-regulation of ΔNp63 in the oesophagus as well as the induction of K14 and the programme of epithelial stratification in the oesophagus.

Other genes involved in the epidermis forming pathway are also worth investigating. These include downstream targets of Bmp such as *Msx1* (Suzuki et al., 1997b) and *Dlx* (Park and Morasso, 2002; Rouzankina et al., 2004). It should be noted that ΔNp63α could also be up-regulated by phosphoinositide 3-kinase pathway (PI3K) through the signal from epidermal growth factor receptor (EGFR) (Barbieri et al., 2003).

7.2.4 p53, p63 and Dnmts

It has been known for some time that *p53* can directly regulate the epigenetic function of *DNA methyltransferases* (*Dnmts*) as well as acting in concert with *Dnmts* (Peterson et al., 2003; Roos et al., 2004; Esteve et al., 2005). It would also be interesting to see if *p63* with the *p53* homologous DNA binding domain have similar functional properties as to interfere or regulate the *Dnmts*. If this is the case, there might be a link between *p63*, *Dnmt* and down-regulation of the columnar genes in the oesophagus epithelium.

7.2.5 Transdifferentiation argument

Is the transition from columnar to stratified squamous epithelium in the oesophagus an example of "transdifferentiation"? It does satisfy the criteria of Eguchi since the starting and finishing phenotypes are well characterised and we have two lines of evidence for a direct conversion of one to the other. Moreover it can occur in the absence of any cell division. However it could also be argued that this is not true transdifferentiation between differentiated tissue types because the columnar cells are simply the normal precursors of the squamous cells and the process is more properly described as a maturation or differentiation event. However, the columnar epithelium can persist, if initiation of the straitified squamous epithelium is blocked. In the *p63* knockout mouse, squamous epithelia cannot be formed and instead the columnar epithelium persists in the oesophagus (and elsewhere) until at least the time of death in the perinatal period (Yang et al., 1999; Mills et al., 1999). For this reason, we consider this process to be reasonably described as "transdifferentiation".

7.2.6 Future perspective

In addition to the data presented in the current thesis, I had also tried to set up *in vitro* model for Barrett's metaplasia and the establishment of a model for the development of the small intestine. This part of work is still ongoing in our lab and the details will not be included.

From the work presented in this thesis, we hope to gain more understanding of the molecular and cellular basis of how the mammalian oesphogeal epithelium develops; the understanding of this process might help us find the molecular or cellular means to stop Barrett's metaplasia from occurring. Consequently, the present study might prove useful for people working in the fields of oesophagus development and Barrett's metaplasia.

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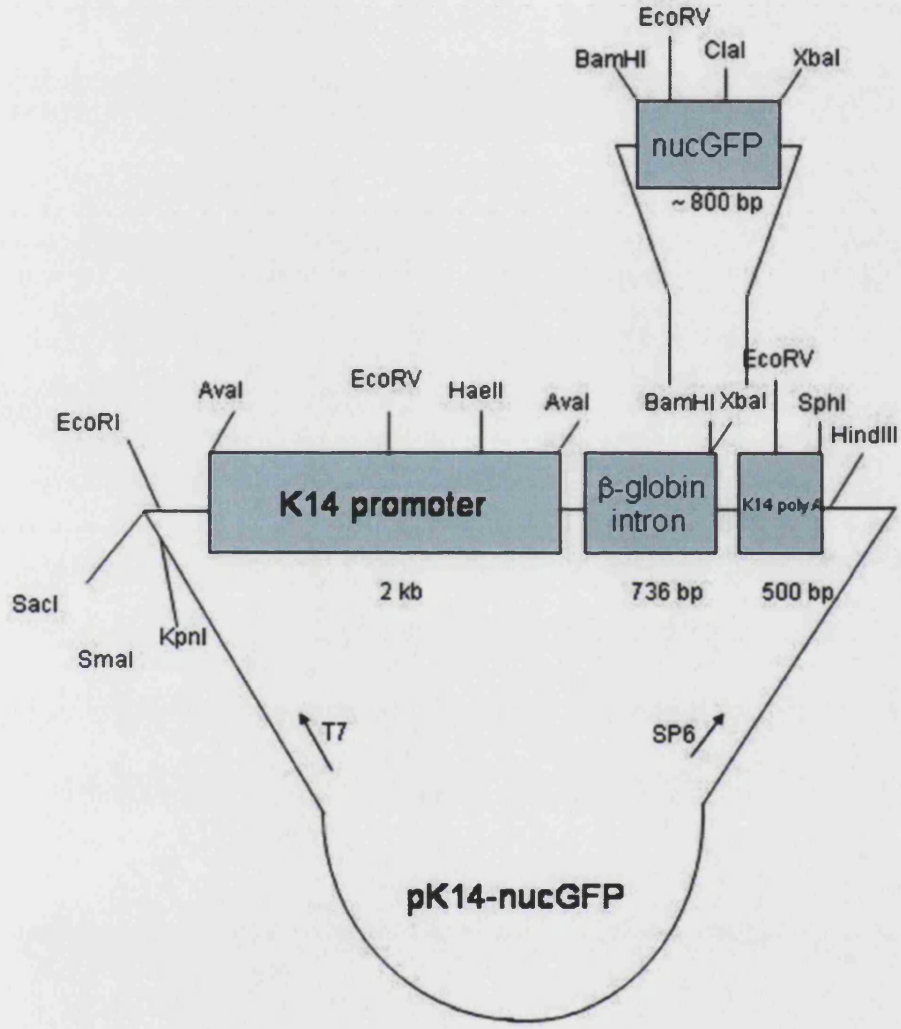
Appendix

A.1 Maps of constructs

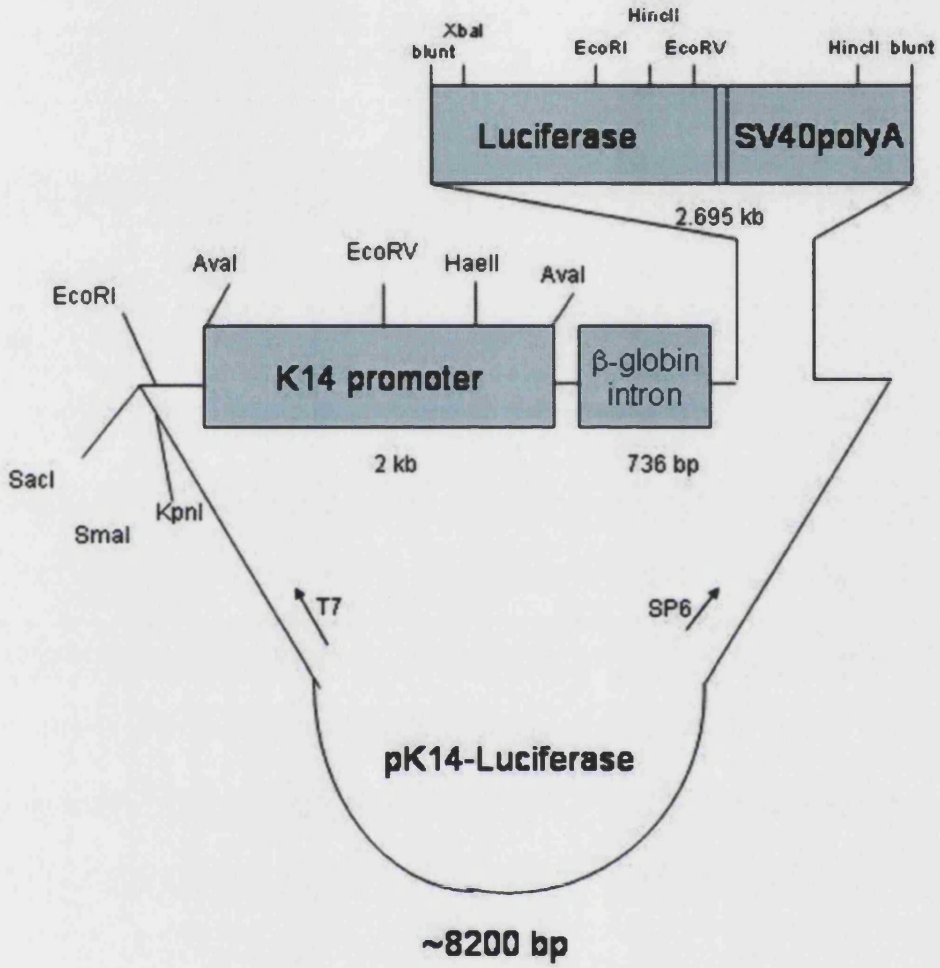
A.1.1 pK14-nucGFP



~ 6779 bp

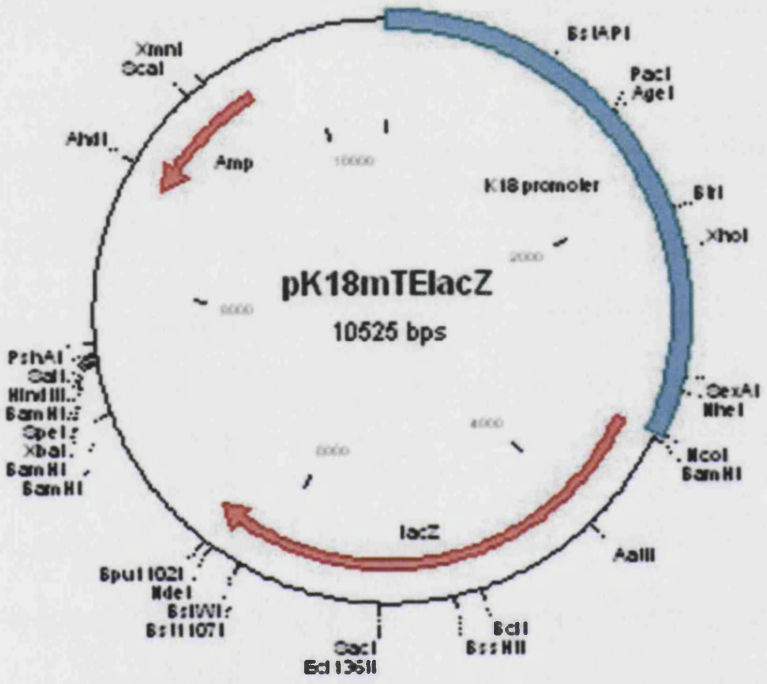
Amp resistance
K14 cassete From: E Fuches

A.1.2 pK14-LucE

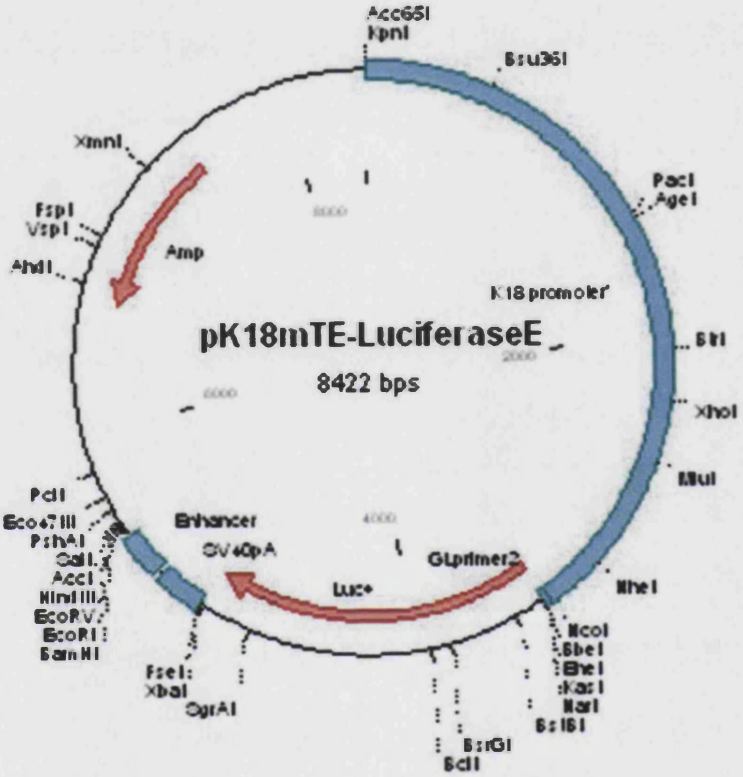


Amp resistance, blunt-blunt cloned
K14 cassete From: E Fuches
Luciferase From: A Ward

A.1.3 pK18-mTEnucLacZ



A.1.4 pK18-mTELucE



A.2 Raw data of FACS analysis

FACS Raw data

Date: 12-04-04	
7d	K14
All Live percentage	30.24
All Dead percentage	69.76
All K14+ percentage	31.12
K14+ Dead	23.96
K14+ Live	7.16
K14+ Dead / All	76.99
	K8
All Live percentage	15.76
All Dead percentage	84.24
All K8+ percentage	50.41
K8+ Dead	37.53
K8+ Live	12.88
K8+ Dead / All	74.45

Date: 16-04-04	
11d	K14
All Live percentage	35.7
All Dead percentage	64.3
All K14+ percentage	37.39
K14+ Dead	20.96
K14+ Live	16.43
K14+ Dead / All	56.06
	K8
All Live percentage	26.76
All Dead percentage	73.24
All K8+ percentage	55.12
K8+ Dead	32.06
K8+ Live	23.06
K8+ Dead / All	58.16

Date: 14-04-04	
15d	K14
All Live percentage	18.4
All Dead percentage	81.6
All K14+ percentage	28.17
K14+ Dead	19.58
K14+ Live	8.59
K14+ Dead / All	69.51
	K8
All Live percentage	20.46
All Dead percentage	79.53
All K8+ percentage	43.4
K8+ Dead	36.69
K8+ Live	16.71
K8+ Dead / All	61.56

Date: 06-04-04	
7d	K14
All Live percentage	18.51
All Dead percentage	81.49
All K14+ percentage	43.13
K14+ Dead	29.73
K14+ Live	13.4
K14+ Dead / All	68.93
	K8
All Live percentage	23.45
All Dead percentage	76.56
All K8+ percentage	40.96
K8+ Dead	24.01
K8+ Live	16.95
K8+ Dead / All	58.62

Date: 28-03-04	
11d	K14
All Live percentage	21.06
All Dead percentage	78.95
All K14+ percentage	18.26
K14+ Dead	12.05
K14+ Live	6.21
K14+ Dead / All	66
	K8
All Live percentage	46.37
All Dead percentage	53.63
All K8+ percentage	54.16
K8+ Dead	10.4
K8+ Live	43.76
K8+ Dead / All	19.2

Date: 01-04-04	
15d	K14
All Live percentage	16.55
All Dead percentage	83.45
All K14+ percentage	29.12
K14+ Dead	18.87
K14+ Live	10.25
K14+ Dead / All	64.8
	K8
All Live percentage	15.4
All Dead percentage	84.59
All K8+ percentage	42.43
K8+ Dead	30.32
K8+ Live	12.11
K8+ Dead / All	71.46

Date: 31-03-04

Date: 19-03-04

Date: 19-04-04

7d	K14
All Live percentage	48.75
All Dead percentage	51.25
All K14+ percentage	26.27
K14+ Dead	7.02
K14+ Live	19.25
K14+ Dead / All	26.72
	K8
All Live percentage	53.83
All Dead percentage	46.17
All K8+ percentage	53.32
K8+ Dead	2.38
K8+ Live	50.94
K8+ Dead / All	4.67

11d	K14
All Live percentage	9.51
All Dead percentage	90.49
All K14+ percentage	19.34
K14+ Dead	11.48
K14+ Live	7.86
K14+ Dead / All	59.36
	K8
All Live percentage	18.07
All Dead percentage	81.92
All K8+ percentage	25.9
K8+ Dead	12.34
K8+ Live	13.56
K8+ Dead / All	47.64

15d	K14
All Live percentage	21.4
All Dead percentage	78.6
All K14+ percentage	12.44
K14+ Dead	10.96
K14+ Live	1.48
K14+ Dead / All	88.1
	K8
All Live percentage	17.74
All Dead percentage	82.26
All K8+ percentage	23.18
K8+ Dead	12.94
K8+ Live	10.24
K8+ Dead / All	55.82

Date: 02-02-04	
7d	K14
All Live percentage	63.85
All Dead percentage	36.16
All K14+ percentage	22.21
K14+ Dead	4.79
K14+ Live	17.42
K14+ Dead / All	21.57
	K8
All Live percentage	32.78
All Dead percentage	67.21
All K8+ percentage	36.33
K8+ Dead	16.05
K8+ Live	20.28
K8+ Dead / All	44.18

Date: 28-02-04	
11d	K14
All Live percentage	73.69
All Dead percentage	26.31
All K14+ percentage	58.67
K14+ Dead	4.57
K14+ Live	54.1
K14+ Dead / All	7.79
	K8
All Live percentage	75.05
All Dead percentage	24.95
All K8+ percentage	81.82
K8+ Dead	13.5
K8+ Live	68.32
K8+ Dead / All	16.5

Date: 03-03-04	
15d	K14
All Live percentage	89.7
All Dead percentage	10.3
All K14+ percentage	92.67
K14+ Dead	5.68
K14+ Live	86.99
K14+ Dead / All	6.13
	K8
All Live percentage	85.12
All Dead percentage	14.88
All K8+ percentage	91.61
K8+ Dead	9.46
K8+ Live	82.15
K8+ Dead / All	10.33

Average for Proportion of Dead Cells

4x

	K14	K14 SE	K14 SE	K8	K8 SD	K8 SE
7d	40.34	20	10.01	31.46	16	8.23
11d	34.99	28	13.97	41.56	25	12.63
15d	36.51	36	17.76	34.68	34	16.85

A.3 Raw data of Luciferase assay

Luciferase assay in Caco-2 and KYSE30 cell line

Cell Line	Reporter	TFs (1 ug each)	Average	SD	SEM
CaCO2	pK18mTELucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	1.39	0.89	0.52
		(pcDNA3)+pC/EBPa	0.93	0.31	0.18
		(pcDNA3)+pC/EBPb	2.53	2.76	1.59
		(pcDNA3)+pTAp63a	0.76	0.25	0.14
		(pcDNA3)+pDNp63a	0.76	0.55	0.27
		(pcDNA3)+pTA*p63a	0.65	0.10	0.06
		pTAp63a+pDNp63a	2.37	3.98	1.99
		pTA*p63a+pDNp63a	0.25	0.06	0.03

Cell Line	Reporter	TFs (1 ug each)	Average	SD	SEM
CaCO2	pK14-LucE	pcDNA3 (2mg)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	1.41	1.00	0.58
		(pcDNA3)+pC/EBPa	0.86	0.62	0.36
		(pcDNA3)+pC/EBPb	1.58	1.08	0.62
		(pcDNA3)+pTAp63a	1.45	0.71	0.41
		(pcDNA3)+pDNp63a	1.93	0.62	0.36
		(pcDNA3)+pTA*p63a	2.19	2.36	1.36
		pTAp63a+pDNp63a	4.32	6.37	3.19
		pTA*p63a+pDNp63a	3.79	4.21	2.43

Cell Line	Reporter	TFs (1 ug each)	Average	SD	SEM
KYSE30	pK18mTELucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	0.87	0.36	0.21
		(pcDNA3)+pC/EBPa	0.81	0.58	0.33
		(pcDNA3)+pC/EBPb	0.39	0.09	0.05
		(pcDNA3)+pTAp63a	0.80	0.62	0.36
		(pcDNA3)+pDNp63a	0.82	0.50	0.29
		(pcDNA3)+pTA*p63a	0.55	0.30	0.18
		pTAp63a+pDNp63a	1.85	3.16	1.58
		pTA*p63a+pDNp63a	0.57	0.50	0.29
KYSE30	pK14-LucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	1.64	0.47	0.27
		(pcDNA3)+pC/EBPa	0.66	0.51	0.30

(pcDNA3)+pC/EBPb	0.47	0.31	0.18
(pcDNA3)+pTAp63a	1.50	1.38	0.80
(pcDNA3)+pDNp63a	1.09	0.46	0.27
(pcDNA3)+pTA*p63a	0.91	0.67	0.39
pTAp63a+pDNp63a	1.81	2.75	1.38
pTA*p63a+pDNp63a	0.51	0.30	0.17

Luciferase assay in OE33 and NIH3T3 cell line

Cell Line	Reporter	TFs (1 ug each)	Average	SD	SEM
OE33	pK18mTELucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	1.33	0.41	0.29
		(pcDNA3)+pC/EBPa	1.24	0.69	0.40
		(pcDNA3)+pC/EBPb	1.40	0.99	0.57
		(pcDNA3)+pTAp63a	0.82	0.16	0.11
		(pcDNA3)+pDNp63a	0.79	0.83	0.41
		(pcDNA3)+pTA*p63a	1.02	0.46	0.27
		pTAp63a+pDNp63a	0.36	0.26	0.13
		pTA*p63a+pDNp63a	0.41	0.23	0.13
OE33	pK14-LucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	1.30	0.72	0.51
		(pcDNA3)+pC/EBPa	0.53	0.23	0.13
		(pcDNA3)+pC/EBPb	0.61	0.38	0.22
		(pcDNA3)+pTAp63a	0.86	0.37	0.26
		(pcDNA3)+pDNp63a	0.44	0.42	0.30
		(pcDNA3)+pTA*p63a	0.53	0.26	0.15
		pTAp63a+pDNp63a	0.42	0.43	0.22
		pTA*p63a+pDNp63a	0.21	0.24	0.14

Cell Line	Reporter	TFs (1 ug each)	Average	SD	SEM
NIH3T3 4.2	pK18mTELucE	pcDNA3 (2ug)	1.00	0.00	0.00
		(pcDNA3)+pAP-2a	2.02	2.50	1.77
		(pcDNA3)+pC/EBPa	1.60	1.03	0.60
		(pcDNA3)+pC/EBPb	1.92	1.81	1.04
		(pcDNA3)+pTAp63a	0.52	0.29	0.21
		(pcDNA3)+pDNp63a	0.51	0.23	0.11
		(pcDNA3)+pTA*p63a	0.58	0.22	0.13
		pTAp63a+pDNp63a	0.65	0.19	0.09

	pTA*p63a+pDNp63a	1.12	0.36	0.21
NIH3T3 4.2 pK14-LucE	pcDNA3 (2ug)	1.00	0.00	0.00
	(pcDNA3)+pAP-2a	2.25	0.41	0.29
	(pcDNA3)+pC/EBPa	1.52	1.06	0.61
	(pcDNA3)+pC/EBPb	2.40	0.77	0.45
	(pcDNA3)+pTAp63a	0.91	0.01	0.01
	(pcDNA3)+pDNp63a	0.92	0.16	0.11
	(pcDNA3)+pTA*p63a	0.81	0.33	0.19
	pTAp63a+pDNp63a	0.83	0.12	0.06
	pTA*p63a+pDNp63a	0.61	0.08	0.04

Luciferase assay-TFs on K18 promoters

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pAP-2a	CaCO2	1.39	0.80
		KYSE30	0.87	0.21
		OE33	1.33	0.29
		NIH3T3 4.2	2.02	1.77

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pC/EBPa	CaCO2	0.93	0.18
		KYSE30	0.81	0.33
		OE33	1.24	0.40
		NIH3T3 4.2	1.60	0.60

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pC/EBPb	CaCO2	2.53	1.59
		KYSE30	0.39	0.05
		OE33	1.40	0.57
		NIH3T3 4.2	1.92	1.04

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pTAp63a	CaCO2	0.76	0.14
		KYSE30	0.80	0.36
		OE33	0.82	0.11
		NIH3T3 4.2	0.52	0.21

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pDNp63a	CaCO2	0.76	0.27
		KYSE30	0.82	0.29
		OE33	0.79	0.41
		NIH3T3 4.2	0.51	0.11

			Average	SEM
pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pTA*p63a	CaCO2	0.65	0.06
		KYSE30	0.55	0.18
		OE33	1.02	0.27
		NIH3T3 4.2	0.58	0.13

			Average	SEM

pK18mTELucE	pcDNA3 (2ug)			1.00	0.00	pK18mTELucE	pcDNA3 (2ug)		1.00	0.00
	pTAp63a+pDNp63a	CaCO2		2.37	1.99		pTA*p63a+pDNp63a	CaCO2	0.25	0.03
		KYSE30		1.85	1.58			KYSE30	0.57	0.29
		OE33		0.36	0.13			OE33	0.41	0.13
		NIH3T3 4.2		0.65	0.09			NIH3T3 4.2	1.12	0.21

Luciferase assays-TFs on K14 promoters

			Average	SEM				Average	SEM
pK14-LucE	pcDNA3 (2ug)		1.00	0.00	pK14-LucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pAP-2a	CaCO2	1.41	0.58		(pcDNA3)+pC/EBPa	CaCO2	0.86	0.36
		KYSE30	1.64	0.27			KYSE30	0.66	0.30
		OE33	1.30	0.51			OE33	0.53	0.13
		NIH3T3 4.2	2.25	0.29			NIH3T3 4.2	1.52	0.61

			Average	SEM				Average	SEM
pK14-LucE	pcDNA3 (2ug)		1.00	0.00	pK14-LucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pC/EBPb	CaCO2	1.58	0.62		(pcDNA3)+pTAp63a	CaCO2	1.45	0.41
		KYSE30	0.47	0.18			KYSE30	1.50	0.80
		OE33	0.61	0.22			OE33	0.86	0.26
		NIH3T3 4.2	2.40	0.45			NIH3T3 4.2	0.91	0.01

			Average	SEM				Average	SEM

pK14-LucE	pcDNA3 (2ug)		1.00	0.00	pK14-LucE	pcDNA3 (2ug)		1.00	0.00
	(pcDNA3)+pDNp63a	CaCO2	1.93	0.36		(pcDNA3)+pTA*p63a	CaCO2	2.19	1.36
		KYSE30	1.09	0.27			KYSE30	0.91	0.39
		OE33	0.44	0.30			OE33	0.53	0.15
		NIH3T3 4.2	0.92	0.11			NIH3T3 4.2	0.81	0.19

			Average	SEM				Average	SEM
pK14-LucE	pcDNA3 (2ug)		1.00	0.00	pK14-LucE	pcDNA3 (2ug)		1.00	0.00
	pTAp63a+pDNp63a	CaCO2	4.32	3.19		pTA*p63a+pDNp63a	CaCO2	3.79	2.43
		KYSE30	1.81	1.38			KYSE30	0.51	0.17
		OE33	0.42	0.22			OE33	0.21	0.14
		NIH3T3 4.2	0.83	0.06			NIH3T3 4.2	0.61	0.04

TF : Luc ratio

			luc/prot/beta-gal	SEM	Normalised Luciferase Activity
KYSE30	pK14-LucE	pcDNA3 1.5 ug (3:1)	0.37	0.07	1.00
		pAP-2a 1.5 ug (3:1)	0.78	0.03	2.13
		pcDNA3 0.5 ug (1:1)	1.04	0.03	1.00
		pAP-2a 0.5 ug (1:1)	1.08	0.08	1.04
	pK18mTELucE	pcDNA3 1.5 ug (3:1)	0.23	0.06	1.00
		pAP-2a 1.5 ug (3:1)	0.62	0.05	2.67
		pcDNA3 0.5 ug (1:1)	0.47	0.07	1.00
		pAP-2a 0.5 ug (1:1)	0.51	0.01	1.08

A.4 List of Materials

A.4.1 Culture Medium

Basal Medium Eagles (BME) with Earle's salts (Sigma)

Minimum Essential Medium with Hank's salts (MEM/Hank's) (Sigma)

Dulbecco minimum essential medium (DMEM) (Sigma)

RPMI-1640 (Sigma)

F-12 HAM Nutrient medium (Sigma)

Hank's Balanced Salt Solution (HBSS) (Sigma)

Foetal Bovine Serum (FBS) (Life Technologies)

Phosphate Buffer Saline (PBSA)

Trypsin / EDTA (Gibco/Invitrogen)

Gentamycin (Gibco/Invitrogen)

Penicillin / streptomycin (Gibco/Invitrogen)

L-Glutamine (Life Technologies)

3 -aminopropyltriethoxysilane (APTES) (Sigma)

Fibronectin (Life Technologies)

1% Non-essential amino acids (NEAA) (Life Technologies)

Keratinocyte serum-free medium (KSFM) (Gibco/Invitrogen)

Supplements for KSFM –

Pituitary gland extract and Epidermal Growth Factor (Gibco/Invitrogen)

A.4.2 Transfection and chemicals

GeneJuice (Novagen)

5-Azacytidine (5-Aza-C) (Sigma)

5-Aza-2 -deoxycytidine (5-Aza-2C) (Sigma)

Mitomycin C (Sigma)

z-VAD-fmk (Caspase Inhibitor VI) (Calbiochem)

1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Vybrant DiI solution) (Molecular Probes)

Cyclopamine (Toronto Research Chemicals)

Ethidium Homodimer (Molecular Probes)

A.4.3 Immunohistochemical and immunostaining reagents

Triton X-100 (Sigma)

Roche Blocking Reagent (Roche)

Antigen Retrieval Citrate buffer (10 x) (LABVISION CORPORATION)

4',6 -diamidino-2-phenylindole (DAPI)

Histoclear (National Diagnostics)

DePeX (BDH)

ABC detection kit (DAKO)

3,3 -Diaminobenzidine (DAB) (Sigma)

Alkaline phosphatase substrate kit (Black) (Vector labs)

5-bromo-4-chloroindol-3-yl β-D-fucopyranoside (X-Fux) (Sigma)

Periodic Acid Solution (50%) (BDH)

Schiff reagent (Sigma)

A.4.4 Luciferase assay

Luciferase Assay Reagent (Promega)

Microplate Luminometer (Berthold LB96V) with Winglow software

Microplate Colorimeter (Anthos AN2001) with Stingray v1.5 software

ONPG (2-nitrophenyl-β-D-galactopyranoside) (Sigma N1127)

Protein Reagent (Biorad 500-0006)

A.4.5 FACS analysis

Cell strainer (40 μm) (Falcon)

Propidium iodide with RNaseA (BD Pharmingen)

Fluorescent activated cell sorting (FACS) machine (BD Coulter flow cytometer)

A.4.6 Electroporation

Micropipette Puller (Sutter, P-97)

Thin wall borosilicate glass capillaries (Clark Electromedical Instrument, GC100-15)

Electrosquareporator (BTX, ECM830)

A.4.7 Microscopes and software programs

Dissecting microscope (Leica Mz12)

Compound microscope (Leica DMRB)

SPOT camera (IMAGE SOLUTIONS) with software

Zeiss LSM 510 confocal microscope (Carl Zeiss) with LSM 5 Image browser program

Photoshop (Adobe System) 6.0

A.4.8 RT-PCR

TRI reagent (Sigma)

RQ-1 DNase (Promega)

Murine Maloney Leukaemia Virus (MMLV) reverse transcriptase (Invitrogen)

DNA thermal cycler (MJ Research Peltier Thermal Cycler-200)

A.4.9 Cell lines and culture conditions

Cell lines (source)	Culture condition
Caco-2 (ECACC)	MEM medium supplemented with 10% FBS, 1% Non-essential amino acids, 2 mM L-Glu and 0.5 U/ml penicillin / 500 ng/ml streptomycin
KYSE30 (ECACC)	50% RPMI-1640 and 50% F-12 HAM Nutrient medium supplemented with 2% FBS, 2 mM L-Glutamine and 0.5 U/ml penicillin / 500 ng/ml streptomycin
OE33 (ECACC)	RPMI-1640 medium supplemented with 10% FBS, 2 mM L-Glu and 0.5 U/ml penicillin / 500 ng/ml streptomycin
NIH3T3 4.2 (ECACC)	DMEM medium supplemented with 10% FBS, 2 mM L-Glu and 0.5 U/ml penicillin / 500 ng/ml streptomycin
HepG2 (ECACC)	DMEM medium supplemented with 10% FBS, 1% Non-essential amino acids, 2 mM L-Glu and 0.5 U/ml penicillin / 500 ng/ml streptomycin

A.4.10 Primary antibodies list

Primary antibody	Species	Dilution	Company
AP-2α	mouse	1:10	DSHB (3B5)
β-catenin	mouse	1:100	Transduction Laboratory (610153)
C/EBPα	rabbit	1:100	Santa Cruz (sc-61)

C/EBPβ	mouse	1:100	Santa Cruz (sc-7962)
Desmin	mouse	1:100	DAKO (D33)
E-cadherin	mouse	1:100	Transduction Laboratory (C20820)
Filagrin	rabbit	1:100	Covance (PRB-417P)
GFP	mouse	1:100	Clontech (8362-1)
GFP	rabbit	1:500	Abcam (ab290)
Involucrin	mouse	1:100	Neomarkers (SY5)
K4	mouse	1:100	Sigma (6B10)
K8	rat	1:200	DSHB (TROMA I)
K10	mouse	1:100	DAKO (DE-K10)
K13	mouse	1:100	Sigma (KS-1A3)
K14	mouse	1:100	NeoMarkers (MS-115)
K14	rabbit	1:100	Covance (PRB-155P)
K18	mouse	neat	Prof. B Lane (LE61)
Ki67	mouse	1:100	BD Pharmingen (556003)
Loricrin	rabbit	1:100	Covance (PRB145P)
Myoglobin	rabbit	1:100	DAKO (A0324)
pan p63	mouse	1:100	Santa Cruz (4A4)
Skeletal myosin (Fast)	mouse	1:100	Sigma (MY-32)
α-smooth muscle actin	mouse	1:100	Sigma (1A4)
Smooth muscle myosin kinase	mouse	1:100	Sigma (K36)

A.4.11 Secondary antibodies list

Secondary antibody	Dilution	Company
FITC anti-mouse IgG	1:100	Vector (FI-2000)
FITC anti-rat IgG	1:100	Vector (FI-4000)
FITC anti-rabbit IgG	1:100	Vector (F-0382)
Texas Red anti-rat IgG	1:100	Vector (TI-9400)
Tetramethylrhodamine Isothiocyanate (TRITC) swine anti-rabbit IgG	1:100	DAKO (R0156)